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Molecular Systematics and Historical Biogeography of the Tree Shrews (Tupaiidae).

Kwai Hin Han
Louisiana State University and Agricultural & Mechanical College

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MOLECULAR SYSTEMATICS AND HISTORICAL BIOGEOGRAPHY OF THE TREE SHREWS (TUPAIIDAE)

A Dissertation

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in

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by
Kwai Hin Han
B. S., Universiti Kebangsaan Malaysia, 1988
M. S., Universiti Kebangsaan Malaysia, 1992
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ABSTRACT

This dissertation examined the molecular systematics and historical biogeography of tree shrews (Tupaiidae). Comparison of cytochrome *b* sequences and DNA hybridization data suggests that cytochrome *b* sequences evolved 7.9 to 13.4 times faster than single-copy nuclear DNA. These two data sets are taxonomically congruent. They indicate that the Mindanao tupaiid *Urogale everetti* is a member of *Tupaia*, and that there is lineage-based variation in rates of evolution among the tupaiids.

A new species of *Tupaia* from Sarawak (Malaysia), *T. stuebingi*, was described using morphological, ecological, and cytochrome *b* sequence data. This lowland species is generally similar in size to the montane species, *T. montana*. Its coat coloration and fur texture resembles the common species, *T. glis*. Cytochrome *b* comparisons show that it is most closely related to *T. montana* at a proportional distance of 11.1%.

Intrafamilial phylogenetic analyses placed the smooth-tailed *Dendrogale* as the outgroup of *Tupaia*, and the bushy-tailed *Urogale everetti* and the pen-tailed *Ptilocercus lowii* as members of *Tupaia*. All five taxa in the *T. glis* species group---*belangeri, chinensis, glis, longipes*, and *salatana*---should be recognized as full species based on their large degree of genetic and morphological differentiation, and their geographic separation.

Maximum likelihood comparisons yielded three distinct tupaiid clades: a basal clade consisting of Asian mainland taxa, a middle clade of Philippine taxa, and a distal

clade of Bornean taxa. This pattern suggests that the Bornean tupaiids originated on the Asian mainland. On Borneo, two phylogeographic patterns---one deep and one shallow---were found, suggesting that the island was colonized repeatedly.

Based on cytochrome *b* data, the sister group of tupaiids is a clade consisting of rabbits, hedgehog, and elephant shrew. However, this sister relationship is likely the result of random long branch attraction between ingroup and distant outgroups. Thus, cytochrome *b* data cannot resolve the interordinal relationships of tupaiids, and their sister group remains unclear.

CHAPTER 1

INTRODUCTION AND BACKGROUND

The tree shrews, or tupaiids, are small (40 to 400 grams), squirrel-like mammals endemic to South and Southeast Asia. They are commonly found from northern India through Borneo and the Philippines (Mindanao, Palawan, Cayo, Calamian, Dinagat, and Siargao islands) and from southern China (Yunan and Hainan) through Indochina, the Malay Peninsula, Sumatra, and Java. The tree shrew family Tupaiidae forms an order of mammals (Scandentia) with no known close relatives (Campbell 1966, Luckett 1980). There are ca. 16 extant species in five genera recognized in this family (Table 1.1).

An early and important classification of Tupaiidae was that of Lyon (1913), who, based on aspects of dentition, skull, and pelage, split the family into two subfamilies: Tupaiinae (*Anathana*, *Dendrogale*, *Tupaia*, and *Urogale*) and Ptilocercinae (*Ptilocercus*). This arrangement was subsequently disputed by Davis (1938), who found numerous cranioskeletal and soft anatomical similarities between *Ptilocercus* and *Dendrogale*. Lyon also placed the large tree shrew (now recognized as *Tupaia tana*) in an additional genus, *Lyonogale*, which was synonymous with *Tana*. Dene et al. (1978) later assigned *Lyonogale* as a subgenus of *Tupaia* based on immunodiffusion evidence. However, Martin (1984) considered *Lyonogale* as a genus consisting of two species, *T. tana* and *T. dorsalis*, presumably based on the presence of a conspicuous mid-dorsal stripe on these two species. *Lyonogale* is now commonly placed within the genus *Tupaia* (Chasen 1940, Honacki et al. 1982, Yates 1984, Corbert and Hill 1992).

Table 1.1—Classification and regional distribution of the Tupaiidae (adapted from Nowak 1991, Han et al. 2000).

Taxonomy	Regional distribution
Subfamily: Tupaiinae	
1. *Anathana ellioti*	northern India
2. *Dendrogale melanura*	Borneo
3. *Dendrogale murina*	eastern Thailand, Cambodia, southern Vietnam
4. *Urogale everetti*	Mindanao, Dinagat, and Siargao islands (Philippines)
Genus: *Tupaia* Raffles, 1821	
Subgenus *Tupaia* Raffles, 1821	
5. *Tupaia nicobarica*	Nicobar Island (Bay of Bengal)
6. *Tupaia javanica*	Java, Sumatra, and Nias islands
7. *Tupaia dorsalis*	Borneo (mainly in Sabah and Sarawak)
8. *Tupaia picta*	Borneo (mainly in Sabah and Sarawak)
9. *Tupaia splendidula*	Borneo (mainly in Kalimantan)
10. *Tupaia gracilis*	Borneo, Bangka, Biliton, and Karimata islands
11. *Tupaia glis**	Borneo, eastern Nepal to southeastern China, Hainan, and the Malay Peninsula, Sumatra, Java and many other nearby islands
Subgenus *Lyonogale* Conisbee, 1953	
12. *Tupaia montana*	Borneo
13. *Tupaia minor*	Borneo, the Malay Peninsula, Sumatra and some nearby islands
14. *Tupaia tana*	Borneo, Sumatra, and some nearby islands
15. *Tupaia palawanensis*	Palawan, Calamian, and Cayo islands (Philippines)
Subfamily: Ptilocercinae	
16. *Ptilocercus lowii*	Borneo, Sumatra, and the Malay Peninsula

*Species complex that includes *belangeri* (Thailand), *chinensis* (Yunan and Hainan), *glis* (the Malay Peninsula), *longipes* (Sabah), and *salatana* (Sarawak)

The tupaiids attain their highest diversity on the island of Borneo where ca. ten species are found, half of them endemic to the island. Because their phylogenetic relationship to other mammal orders is obscure and intriguing, tree shrews have been subject to numerous morphological studies investigating their higher level phylogeny (e.g., Lyon 1913, Le Gros Clark 1924, 1925, Davis 1938, McKenna 1963, Campbell 1966, 1974, Martin 1968, Butler 1972, Steele 1973, Luckett 1980). However, molecular data on tree shrews, particularly at the intrafamilial level, remain sparse and have accumulated slowly despite the recent blooming of DNA technology.

There are only two major molecular studies of tree shrews at the intrafamilial level. Using immunodiffusion, Dene et al. (1978) studied the interspecific relationships of 10 tupaiid species in three genera (*Ptilocercus*, *Tupaia*, and *Urogale*). They separated the genus *Tupaia* into two subgenera: *Lyonogale* and *Tupaia*. They also recognized the species *T. belangeri*, *T. chinensis*, and *T. longipes*, although these three taxa often are regarded as subspecies of *T. glis* (Napier and Napier 1967). Based on DNA hybridization comparisons, Han et al. (2000) investigated the interspecific relationships and biogeography of six species, while trying to track the evolution of tree shrews in Borneo. These two studies, generally congruent in elucidating the interspecific relationships of tree shrews, suffer from two chronic problems, namely a lack of an unambiguous outgroup, for rooting the molecular tree, and missing taxa.

Several factors complicate the study of tupaiid intrafamilial relationships (Han et al. 2000). First, because Tupaiidae is widely distributed across several countries in Southeast Asia, it is difficult to obtain a full range of specimens for

study because of local and regional social-political instability. To help solve these problems, I have recently developed the necessary laboratory protocols for obtaining partial sequences from museum study skins. However, these skins are very rare and museums were willing to loan me some but not all the species I needed because of their destructive sampling policies. Second, due to the absence of a fossil record (Van Valen 1965) and a close outgroup, it is difficult to root the tupaiid tree, study character state polarity, or date the group's time of origin. Third, sea level changes associated with Tertiary and Quaternary ice ages created repeated opportunities for invasion and isolation of the various islands in the Malay Archipelago, or Sundaland (e.g., Borneo, Java, Palawan, Sumatra). Thus, physically juxtaposed taxa are not necessarily closely related, particularly in Borneo. Fourth, the tree shrews as a group appear to be an extremely old taxon, and a genetic marker is required to determine the relationship between close species and ancient ancestors simultaneously; but such marker has not been found. These problems will probably continue to haunt the study of tupaiid phylogenetic relationships for many years to come.

Borneo has a well documented but complex history of land bridges connecting and disconnecting it from its neighboring islands (Java, Palawan, and Sumatra) and mainland Asia. Palaeogeological evidence (Metcalfe 1988 and references therein) indicates that large islands lying on the Sunda shelf (Borneo, Java, and Sumatra) are composite structures, consisting of at least four major allochothonous terranes (Indochinese, Sibumasu, East Malayan, and southwest Bornean, Figure 1.1). Sundaland was formed by the accretion of continental plates during Paleozoic and Mesozoic, which subsequently drifted apart, reoriented and

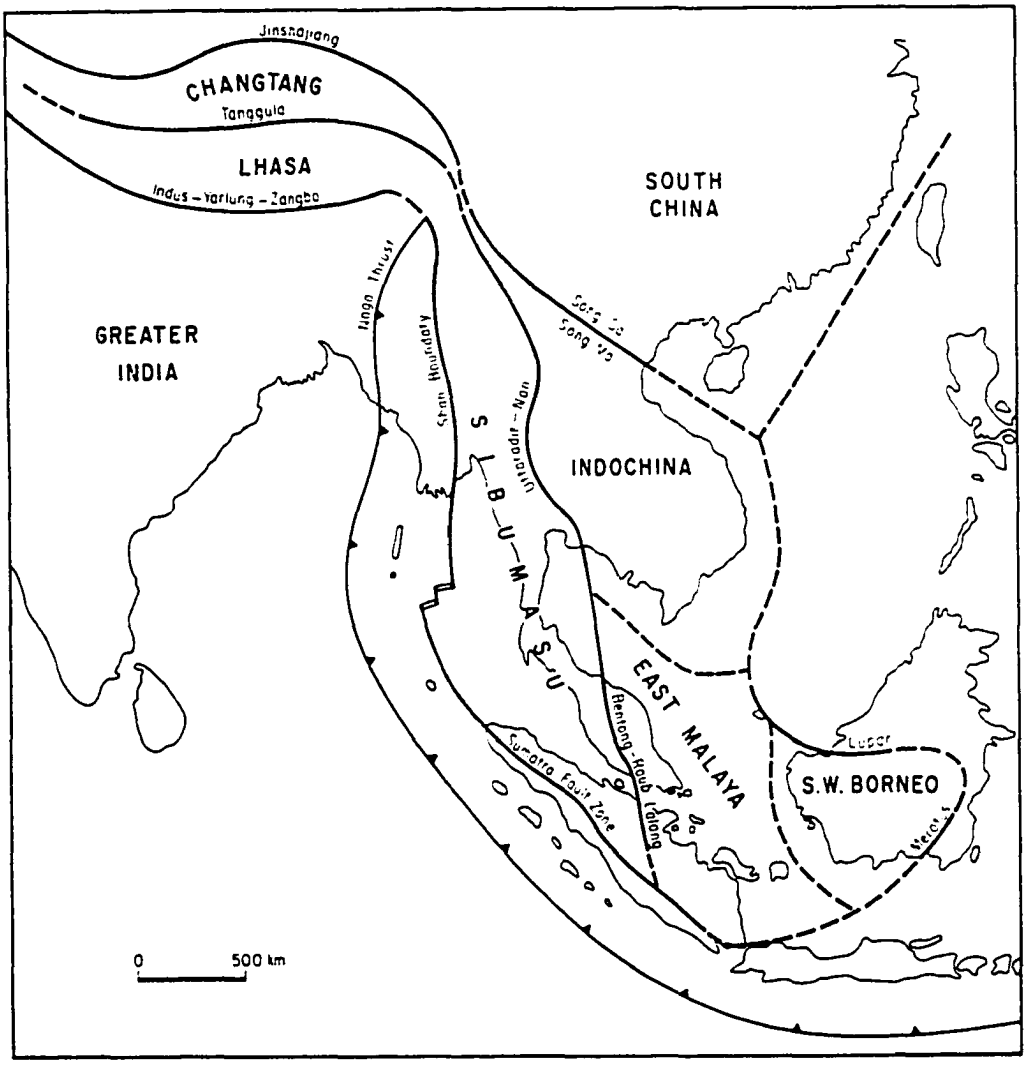

Figure 1.1---The tectonostratigraphic terranes of Southeast Asia (adapted from Metcalfe 1988).

collided again. High ground exceeding 1,000 m has existed in the Malay Peninsula since the Paleozoic and in southwest Borneo since the Mesozoic (Holloway 1986).

Of particular importance to the development of flora and fauna were cyclic sea-level changes associated with ice ages and other geological fluctuations occurring during the mid-Oligocene (25-30 million years ago) and the Pleistocene (18,000 to 2 million years ago). These created repeated opportunities for invasion, colonization, and isolation of floral and faunal populations in Borneo (Audley-Charles 1987). The connection and disconnection of these islands coupled with the isolation afforded by Wallace's line demarcating the separation of Asian (Sunda) and Australian (Sahul) continental plates at the eastern edge of Borneo, generated a land-bridge speciation pump. Within the island of Borneo, the numerous highlands (up to 4,100 m on Mount Kinabalu) and several large river systems formed natural barriers to the spread of various vertebrate groups, particularly small mammals, which have a relatively low dispersal ability. These barriers further intensified the effects of Borneo's periodic isolation. Together, the speciation pump and Borneo's unique geography gave rise to an unusually high biodiversity on the island. The result is a complex pattern of widespread and endemic taxa.

I present in this dissertation a study of the molecular systematics and historical biogeography of tree shrews using mitochondrial cytochrome *b* DNA sequences. Given the limitation in taxic sampling, I focus mainly on the divergence of tree shrews between Borneo and the Asian mainland (Malaya). Preliminary analysis of cytochrome *b* sequence variation suggested that the Malaya, Sabah, and Sarawak populations of *T. glis* are distantly diverged, to the extent of being separate

species with proportional pairwise distances ranging from 8% to 19%. Because of these great distances, I am using a phylogenetic rather than population genetic approach to study them.

In the course of these cytochrome *b* comparisons, I draw upon my previous studies of the population genetics (Han 1991) and biogeography of tree shrews in Sabah (Han et al. 2000). Previously, using allozyme and DNA hybridization data augmented by information on sea-level changes, I proposed a model of speciation occurring at the interface of lowland and montane zones to explain the origin, distribution, and endemism of the Bornean montane tree shrew, *Tupaia montana*.

The structure of this dissertation is as follows. In Chapter 2, I compare cytochrome *b* sequence and single-copy nuclear DNA hybridization data. This comparison provides insight into the rates of codon site changes, transitional versus transversional changes, saturation, and the overall pattern of mitochondrial versus single-copy nuclear evolution. An understanding of these patterns is essential to choosing an appropriate model for building trees from cytochrome *b* data. These patterns may also shed light on the rate of tree shrew evolution, particularly whether the tree shrews are morphologically static or molecularly volatile.

In Chapter 3, using ecological, morphological, and molecular data, I describe a new species of *Tupaia* based on three adult specimens that I collected from Lanjak-Entimau Wildlife Sanctuary, Sarawak, in 1997. Cytochrome *b* sequence comparisons among eight taxa indicate that this species is most closely related to *T. montana*, but at the relatively large proportional distance of 11.1%.

My previous work also suggested that Tupaiidae in Borneo may have dispersed from the mainland and undergone *in situ* speciation, and the species complex of the common tree shrew *Tupaia glis* appears to be much older than we previously believed (Han et al. 2000). The mainland taxa (*T. glis* in Malaya, *T. belangeri* in Thailand and Indochina, and *T. chinensis* in southern China) differ from the island taxa in body size, and they appear to be ancestral to the Bornean taxa (*T. longipes* in Sabah and *T. salatana* in Sarawak). In Chapter 4, I investigate whether the island-dwelling populations of *T. glis* arrived just once before Borneo was disconnected from the Asian mainland, or repeatedly, i.e., whether *T. glis* reinvaded Borneo during the ice ages of the Tertiary (mid-Oligocene, 25-30 million years ago) and again in the Quaternary (18,000 to 2 million years ago). I also assess whether the cytochrome *b* data generated in this study would be able to provide adequate phylogenetic signal to solve the interordinal relationships of tree shrews to other eutherian mammals.

These chapters are followed by a brief summary and conclusion, in which I attempt to interpret historical patterns of tree shrews in light of molecular evolution. I also identify future research needs, which include using longer sequences such as the complete mitochondrial genome, or several nuclear genes, for phylogeny reconstruction.

CHAPTER 2

PATTERNS OF INTERSPECIFIC DIVERGENCE AND RELATIVE RATES OF EVOLUTION AMONG THE TREE SHREWS: A COMPARISON OF CYTOCHROME *B* AND DNA HYBRIDIZATION DATA

INTRODUCTION

The tree shrews, or tupaiids (Tupaiidae), were once known as the missing link between the insectivores and the primates (Martin 1966) because of their morphological intermediacy: they are insectivorous in habit, yet they possess scrotums for testes in males and the orbits of their skulls are encircled by bone, as in primates. Despite the fact that the tree shrews are now relegated to their own order, the Scandentia (Wagner cited in McKenna 1975), the notion that they are a form of primitive primate (Sorenson 1970) suggests that the tree shrews could be a very old taxon. Based on DNA differences, researchers have recently suggested that modern mammals, including primates and tree shrews, may have emerged as early as 100 million years ago (Normile 1998).

Based on allozyme data (Han 1991), DNA hybridization data (Han et al. 2000), and information on sea-level changes, I have suggested that the montane tree shrew, *Tupaia montana*, evolved from a lowland progenitor at least as early as the mid-Oligocene (ca. 25-30 million years ago). Endemic to the island of Borneo, *T. montana* is one of the two most recently derived—i.e., youngest—tupaiid species (Dene et al. 1978, Han et al. 2000).

Interestingly, despite ancient origins and considerable genus-level diversity (five genera, Table 1.1), members of Tupaiidae exhibit little variation in external morphology. Most strikingly, the ca. 11 species comprising the largest and the most

widespread genus, *Tupaia*, are distinguished mainly by small differences in body size, pelage color, and presence or absence of a longitudinal mid-dorsal stripe. This paradox of morphological stability with considerable genetic divergence suggests that the tree shrews have either evolved rapidly at the molecular level or they have existed for a long time in a very specialized, stable niche.

Even at the intrafamilial level, tree shrews show tremendous genetic divergence, which has led to problems in assigning genera and designating species. For example, the monotypic Mindanao tree shrew, *Urogale everetti* (called *Urogale* hereafter), is generally presumed to be an outgroup of *Tupaia*. However, when *Urogale* was compared to other *Tupaia* species using DNA hybridization, it lay on a substantially shorter branch than the common tree shrew, *T. glis* (Han et al. 2000). *Tupaia glis*, because of its widespread distribution throughout Southeast Asia, is presumably ancestral to other *Tupaia* species. This branch length anomaly suggests either that (1) *Urogale* may in fact be a member of *Tupaia* or (2) the rate of molecular evolution in *Tupaia* and *Urogale* varies to a large degree. Unfortunately Han et al. (2000) were not able to examine the relative rates of *Urogale* and *Tupaia* evolution because, with no tissues of other tupaiid genera available, they lacked an unambiguous outgroup, and species in other orders are too distant to form DNA hybrids. Also, in the absence of fossil records, it was impossible to determine the absolute date of *Urogale* (or any tree shrews) divergence. The notion that the Tupaiidae is either very old or that it exhibits a fast rate of evolution is further supported by the cytochrome *b* sequence data obtained in the present study. These

data vary substantially among species of tree shrews, with uncorrected proportional distances ranging from 11.61% to 18.69%.

In this chapter, I compare cytochrome *b* sequence and DNA hybridization data obtained from the same six tree shrew species: *Tupaia glis longipes, T. gracilis, T. minor, T. montana, T. tana,* and *Urogale everetti*. Cytochrome *b* is a mitochondrial gene whereas DNA hybridization assesses the genetic divergence of single-copy nuclear DNA (scn-DNA). Because mitochondrial and nuclear genes occupy different genomes, genetic distances derived from these two data sets are largely independent (Sheldon et al. 2000). These distances can be compared to one another to study relative rates and patterns of nuclear and mitochondrial DNA (mtDNA) evolution. Assessment of phylogenetic congruence between scn- and mtDNA helps to separate the phylogenetic signals in the mtDNA from the noise (mostly caused by saturation). Identification of the most informative mtDNA characters should help produce a better phylogenetic estimate, which can then be used for biogeographic and other evolutionary analyses.

DNA hybridization data have several advantages over cytochrome *b* sequences. Because scn-DNA evolves more slowly than mtDNA, it is largely free of saturation (Sheldon and Bledsoe 1989) at the levels of tree shrew divergence. Thus, on rate graphs, DNA hybridization distances provide a fairly linear time scale for studying genetic divergence of the fast-evolving cytochrome *b* data (Sheldon et al. 2000). Also, fluctuation in genetic distances due to sampling bias is minimal in DNA hybridization because it measures a large number of nucleotide substitutions simultaneously (Werman et al. 1996).

One advantage of cytochrome *b* sequences relative to DNA hybridization data is that they may be able to resolve relationships among closely related species more effectively. Also, as opposed to the distance data of DNA hybridization, cytochrome *b* data are discrete characters that can be partitioned into various data subsets and analyzed via a wider array of phylogenetic methods, including maximum likelihood and parsimony. Thus, DNA hybridization and cytochrome *b* data sets complement each other, one data set making up for the weaknesses in the other. Taken together, they provide substantial insight into the phylogeny and molecular evolution of the Tupaiidae.

MATERIALS AND METHODS

Selection of taxa

The same six species used in DNA hybridization comparisons (Han et al. 2000) were sequenced for the entire cytochrome *b* gene (see the Appendix).

PCR amplification and DNA sequencing

Standard phenol-chloroform extractions (Hillis et al. 1996) were performed after digestion of 0.1 g liver tissues with Proteinase K (25 μl, 10 mg/ml) in 500 μl STE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 7.5) and 25 μl 20% SDS at 55°C for two hours. DNA was then precipitated in 50 μl 3M sodium acetate (pH 5.2) and 1 ml chilled absolute ethanol. Upon spinning, draining, and drying, DNA was suspended in 250 μl of sterile Nanopure water. The entire cytochrome *b* was amplified, in two pieces, using the following primer pairs: (1) L14724: 5'-TCAAAGCTTACACCAGTCTTGTAAACC-3' (Irwin et al. 1991) and H15560: 5'-SGCAAATARGAARTATCATTC-3', which was modified from Palumbi (1996);

and (2) L403: 5'-TGAGGACAAATATCCTTC-TGAGG-3' and H16065: 5'-GGAGTCTTCAGTCTCTGGTTTACAAGACC-3' (Helm-Bychowski and Cracraft 1993). "L" and "H" refer to heavy- and light-strand primers. Primer L403 is a reversed compliment of H15149 (Kocher et al. 1989). Each primer pair amplified a fragment of ca. 750 base pairs. PCR reactions for each primer pair were carried out in a GeneAmp PCR System 2400 oil-free thermocycler (Perkin Elmer), using a 50 µl-reaction volume containing 0.5 µM of each primer, 10 mM of each dNTPs, 2.5mM $MgCl_2$, and 1.25 U Taq polymerase (Perkin Elmer). An annealing temperature of 50°C was used in PCR reactions for all taxa except *T. montana*, which was annealed at 51°C. Thermal cycling was as follows: 30 cycles with denaturation at 94°C for 30 s, annealing at 50°C to 51°C, and extension at 72°C for 30 s. These cycles were followed by a final extension at 72°C for 7 min.

PCR products were electrophoresed in a 1% agarose gel at 70 V for 45 min, stained with 10 mg/ml ethidium bromide, excised, and purified using QIA quick Gel Extraction Kit (QIAGEN, #28704). Both strands of the PCR product were sequenced using a BigDye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems), followed by sequencing in an ABI 377 automated sequencer (Perkin Elmer Applied Biosystems) with a 5% Long Ranger (FMC) gel. For primer pair L14724 and H15560, an internal heavy-strand primer modified from H15149 (Kocher et al. 1989), 5'-CCTCAGAAKGATATYTGHCCTC-3', was used.

Data analysis

All individuals were sequenced completely in both directions for the entire cytochrome *b* gene. Light- and heavy-strand sequences were aligned, using

Sequencher 3.1 (Gene Codes Corporation) and MacClade 3.0 (Maddison and Maddison 1992), with sequences of human, lemur (*Lemur catta*), shrew (*Sorex araneus*), rabbits (*Lepus sinensis* and *Oryctolagus cuniculus*), and pig (*Sus scrofa*) downloaded from GenBank (accession numbers in Appendix). Sequence alignments were unambiguous. Nuclear pseudogenes seemed not to be a problem because internal stop codons, insertions, and deletions were absent. Moreover, BLAST search in GenBank gave pig cytochrome *b* as the closest match to tree shrews with >20% divergence, suggesting no contamination of samples with known DNA.

Computer packages MEGA version 1.02 (Kumar et al. 1993) and PAUP*4.0b1 (Swofford 1998) were used to estimate the following features of the sequences: base frequencies, uncorrected pairwise distances (p-distance), and uncorrected ratio of transitions to transversions. The skewness of tree-length distribution was examined based on 10,000 random trees using G statistics (Hillis and Huelsenbeck 1992) implemented in PAUP*. Distances were corrected with PAUP* based on the most appropriate maximum likelihood model determined using ModelTest (Posada and Crandall 1998). The designated model was gamma-General Time Reversible-invariable sites (GTR+I+Γ) with the following parameters: A-C 1.8301, A-G 5.2067, A-T 1.6081, C-G 0.1519, and C-T 9.3035. The proportion of invariable sites (I) was 0.5895, and the shape parameter of the gamma distribution (α) was 3.399. The maximum likelihood tree was bootstrapped (100 replications) using the UNIX version of PAUP*.

Following Sheldon et al. (1999, 2000), cytochrome *b* data were subdivided into transitions and transversions according to codon site positions, generating six

partitions. Uncorrected and corrected distances of the partitioned and unpartitioned data of cytochrome *b* were graphed as proportional nucleotide divergence versus DNA hybridization distance to examine the effects of saturation on nucleotide substitutions. DNA hybridization distance was expressed as proportional delta Tmode (delta Tmode values divided by 100) because 1 unit of delta Tmode generally approximates 1%-sequence divergence (Springer et al. 1992, Sheldon et al. 2000). The intraspecific divergences of four tree shrew species, *T. glis longipes*, *T. minor*, *T. montana*, and *T. tana*, were included among both cytochrome *b* and DNA hybridization distances to define the initial slopes of various graphs. DNA hybridization distances were not corrected because Tmode is determined by the position of the peak in the melting curve of DNA hybridization and is relatively unaffected by the low-temperature tail of the melting distribution. Thus, delta Tmode is less affected by compression than other measures such as Tm (Sheldon and Bledsoe 1989, Springer and Kirsch 1991, Slikas 1997). In addition, DNA hybridization distances among species with less than ca. 15% nuclear divergences are not very strongly affected by saturation (Sheldon and Bledsoe 1989).

Differences in topology among trees derived from DNA hybridization and cytochrome *b* data were tested quantitatively, using the Kishino-Hasegawa method (1989) implemented in PAUP* under the framework of maximum likelihood. Two topologies of the DNA hybridization data were tested: (1) a 50%-majority rule consensus tree with *Urogale* as the outgroup (Han et al. 2000), and (2) a DNA hybridization tree rooted with *T. glis* (Han et al. unpublished data). Relative to the root, *T. gracilis* lies distal to *Urogale* and *T. glis* in (1), and it is basal to *Urogale* but

distal to *T. glis* in (2). Because there were fewer taxa examined by DNA hybridization than by cytochrome *b* sequencing, these topology analyses were limited to the taxa for which both kinds of data were available. Parsimony trees of cytochrome *b*, generated in PAUP* via heuristic searches, with tree-bisection-reconnection branch-swapping and MULPARS options in effect under various Ti:Tv ratios (1:1 to 9:1), were also compared against the maximum likelihood cytochrome *b* tree. Molecular clock was not assumed in these searches.

RESULTS AND DISCUSSION

Characterization of cytochrome *b* data

Each aligned tree shrew cytochrome *b* sequence consisted of 1140 base pairs. Of the 383 variable sites, 322 were potentially parsimony-informative. Table 2.1 lists the distribution of the variable sites and parsimony-informative sites and the average nucleotide composition of the tree shrews based on codon-site partitions. The nucleotide composition was fairly evenly distributed at first-codon positions but less so at second and third positions. These patterns conform to the composition of the cytochrome *b* gene in other mammals (Irwin et al. 1991), suggesting that no pseudo- and non-homologous genes were amplified in this study. Table 2.2 presents the interspecific genetic distances of the tree shrews derived from cytochrome *b* and DNA hybridization. Cytochrome *b* distances less than 10% most likely represent within-species variation and will not be discussed in this chapter.

Relative rates of evolution

When codon-site partitions of cytochrome *b* p-distances are plotted against the DNA hybridization distances (Figures 2.1 and 2.2A), there appear to be three

Table 2.1---Variability of cytochrome *b* sequences partitioned by sites and codon positions.

						Variable sites	Parsimony-informative sites
	A	T	C	G	Total sites	% (#)	% (#)
1st position	27.4%	22.7%	25.4%	24.5%	380	15.67 (60)	13.04 (42)
2nd position	20.8%	40.6%	24.5%	14.2%	380	5.48 (21)	4.35 (14)
3rd position	40.0%	19.2%	37.6%	3.3%	380	78.85 (302)	82.61 (166)
All positions	29.4%	27.5%	29.2%	14.0%	1140	Total (383)	Total (322)

Table 2.2---Cytochrome *b* and DNA hybridization distances for six tree shrew species.

	Cytochrome *b* distances*		DNA hybridization distances**
Pairwise comparisons	p-distance	ML distance	proportional delta Tmode
T. montana x *T. montana*	0.0062	0.0063	0.0003
T. tana x *T. tana*	0.0247	0.0390	0.0044
T. glis x *T. glis*	0.0520	0.0651	0.0046
T. minor x *T. minor*	0.0535	0.0627	0.0094
T. montana x *T. tana*	0.1161	0.1620	0.0098
T. montana x *T. minor*	0.1376	0.2150	0.0159
T. tana x *T. minor*	0.1352	0.2117	0.0179
T. tana x *T. gracilis*	0.1583	0.3028	0.0280
T. montana x *U. everetti*	0.1577	0.2877	0.0287
T. montana x *T. gracilis*	0.1653	0.3105	0.0290
U. everetti x *T. minor*	0.1750	0.3301	0.0297
U. everetti x *T. gracilis*	0.1754	0.3565	0.0298
U. everetti x *T. tana*	0.1472	0.2653	0.0320
T. gracilis x *T. minor*	0.1333	0.2293	0.0339
T. glis x *U. everetti*	0.1760	0.3418	0.0385
T. glis x *T. montana*	0.1869	0.3686	0.0394
T. glis x *T. tana*	0.1730	0.3292	0.0403
T. glis x *T. gracilis*	0.1662	0.3346	0.0406
T. glis x *T. minor*	0.1725	0.3336	0.0428

*p-distance = proportional (uncorrected) distance; ML distance = maximum likelihood distance under the GTR+I+Γ model.

**expressed as proportional delta Tmode (delta Tmode values divided by 100) because one unit of delta Tmode generally approximates 1%-sequence divergence (Springer et al. 1992, Sheldon et al. 2000).

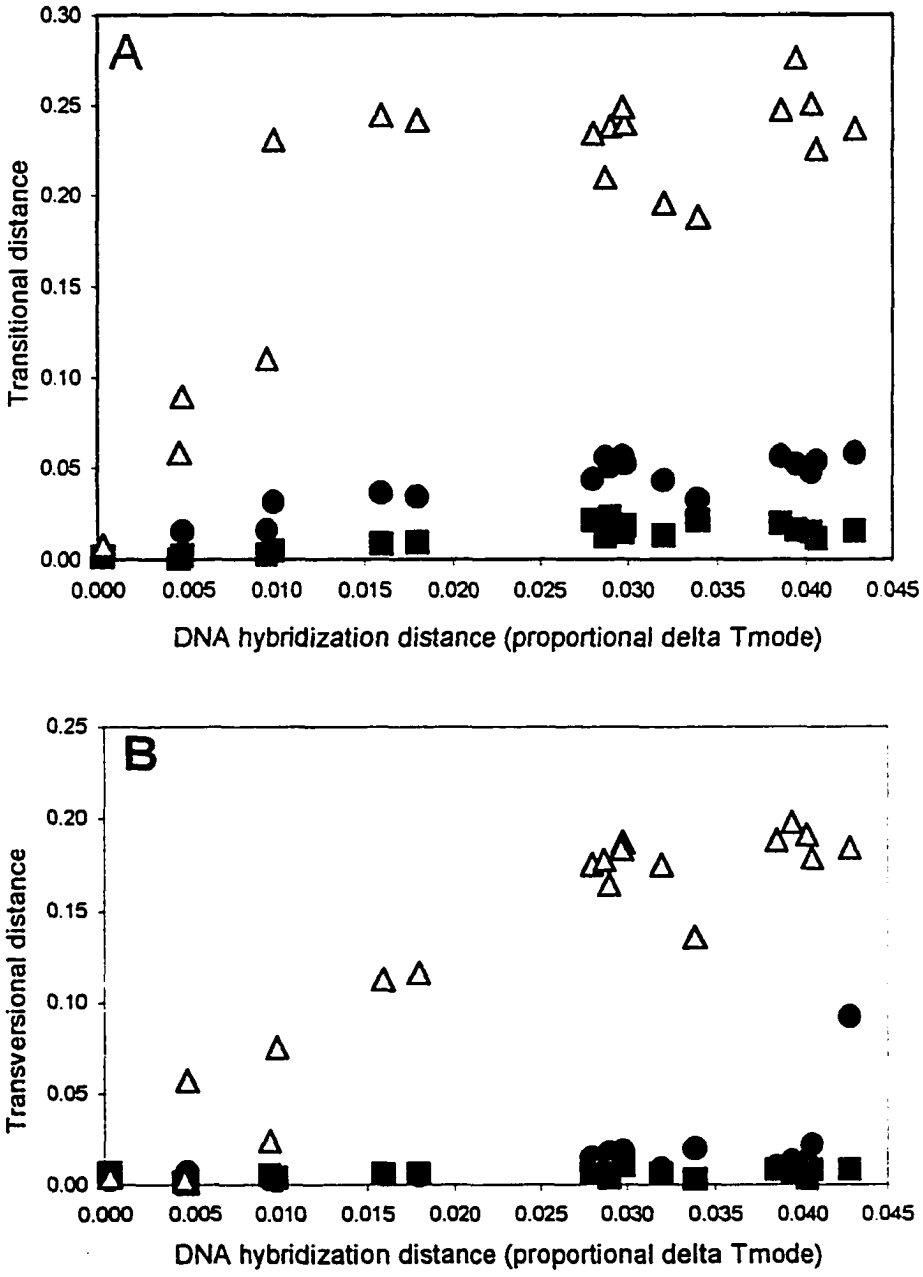

Figure 2.1—Pairwise divergences of cytochrome *b*, by codon position, versus DNA hybridization distances. (A) Transitions and (B) Transversions (circles are first-, squares are second-, and triangles are third-position distances).

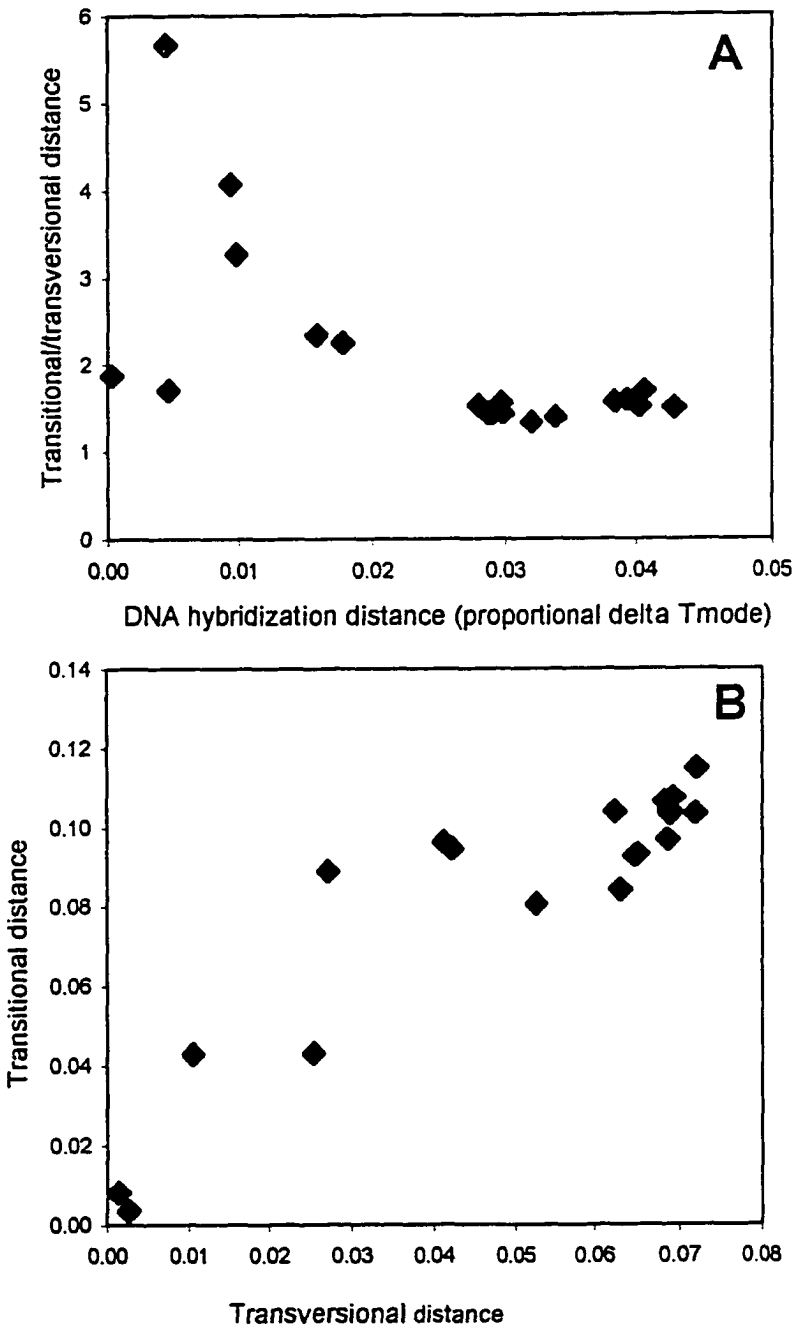

Figure 2.2---Transitional:Transversional (Ti:Tv) analysis. (A) Uncorrected pairwise Ti to Tv ratios of cytochrome *b* data versus DNA hybridization distances. (B) Proportion of uncorrected pairwise Ti versus proportion of pairwise Tv of cytochrome *b* data.

distinct clusters of points: (1) between delta Tmode 0.00 to 0.02; (2) between delta Tmode 0.025 to 0.035, and (3) above delta Tmode 0.037. However, only two clusters are distinct in the Ti:Tv plot of cytochrome *b* distances (Figure 2.2B). This distance compression suggests that the uncorrected cytochrome *b* data are heavily saturated.

Interestingly, when the uncorrected third-position transversional distances are plotted against the corrected total (maximum likelihood) distances, three clusters of points again are apparent (Figure 2.3), with an expected R^2=0.97 for a linear regression, which is slightly better than the plot of transversional distances against DNA hybridization value (expected R^2=0.87, Figure 2.1B). The GTR+I+Γ model appears to be effective in removing a large portion of the saturation. However, the high correlation in the third-position transversional distances against the maximum likelihood distances (Figure 2.3) is partly because the third-position transversional distances are a subset of the total distances. Similar to the findings of other studies (Irwin et al. 1991, Sheldon et al. 2000), third-position transversions in tree shrews are potentially highly phylogenetically informative.

The interspecific divergence of uncorrected cytochrome *b* distances spans a somewhat narrow range, from 11.61% to 18.70% (Table 2.2, Figure 2.4A). The curve levels off at a proportional distance of ca. 14%. Maximum likelihood correction substantially improves the linearity of the cytochrome *b* distances against DNA hybridization distances, but the line begins to plateau when delta Tmode values exceed 0.03. The curve of the corrected maximum likelihood distances (Figure 2.4A) is apparently curvilinear, but when it is linearly regressed (Figure 2.4B), a

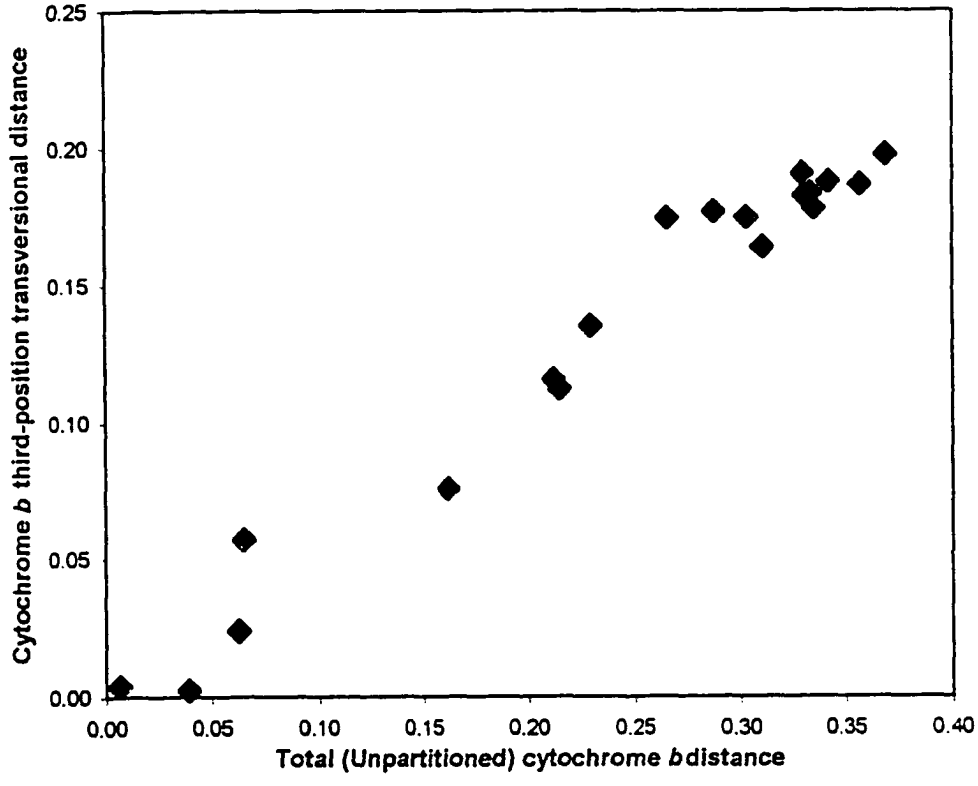

Figure 2.3---Distances based on cytochrome *b* third-position transversions versus maximum likelihood distances based on the GTR+I+Γ model. Expected regression is y=0.5634x-0.0021, and R^2=0.97.

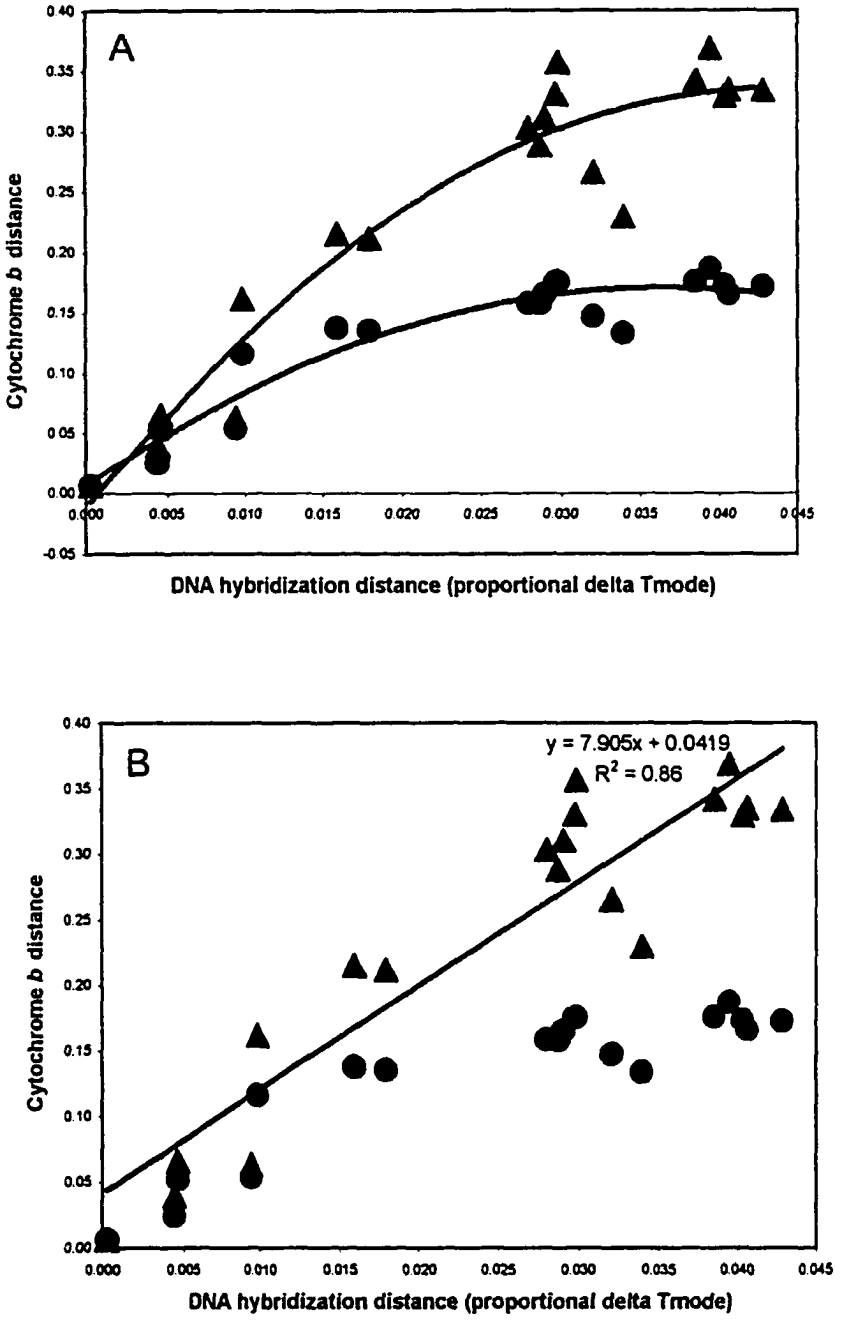

Figure 2.4---Cytochrome *b* sequence distances versus DNA hybridization distances. (A) Polynomial fitting. (B) Expected linear regression. Triangles represent maximum likelihood (ML) distances (GTR+I+Γ model) and circles represent uncorrected proportional distances.

reasonably high expected R^2 value (0.86) is obtained with a slope of 7.91, suggesting that the cytochrome *b* gene evolves 7.91 times faster than the scn-DNA. However, this slope probably underestimates the relative rate of the cytochrome *b* gene because it shows a positive y-intercept instead of passing through the origin. This pattern suggests that a certain amount of saturation may exist in the cytochrome *b* data even among closely related taxa, and despite the maximum likelihood correction.

To investigate the initial rate of cytochrome *b* divergence in more detail, I partitioned the plot of cytochrome *b* versus DNA hybridization distances by removing data points that are apparently saturated, i.e., data points with proportional delta Tmode values exceeding 0.0159 (Table 2.2, Figure 2.4A). I chose 0.0159 as the cut-off for two reasons. First, there is a 0.2% difference in proportional delta Tmode values between 0.0159 and the next data point, 0.0179 (divergence between *T. minor* and *T. tana*), and the curve of the uncorrected cytochrome *b* distances (Figure 2.4A) shows that the point 0.0179 lies on the saturation plateau. Second, when the point 0.0179 is included in a partitioned plot of uncorrected cytochrome *b* versus DNA hybridization distances, its intercept (0.0048, graph not shown) deviates farther from the origin than when it is excluded (0.001, Figure 2.5).

The regression between uncorrected cytochrome *b* and DNA hybridization distances in the partitioned plot (Figure 2.5) shows that cytochrome *b* sequences evolve 8.65 times faster than the scn-DNA. Given that the initial slope is fairly well defined with four data points of intraspecific divergences, this estimate seems reasonable. Under the assumption of a constant evolutionary rate, the regression between proportional cytochrome *b* and DNA hybridization distances in the

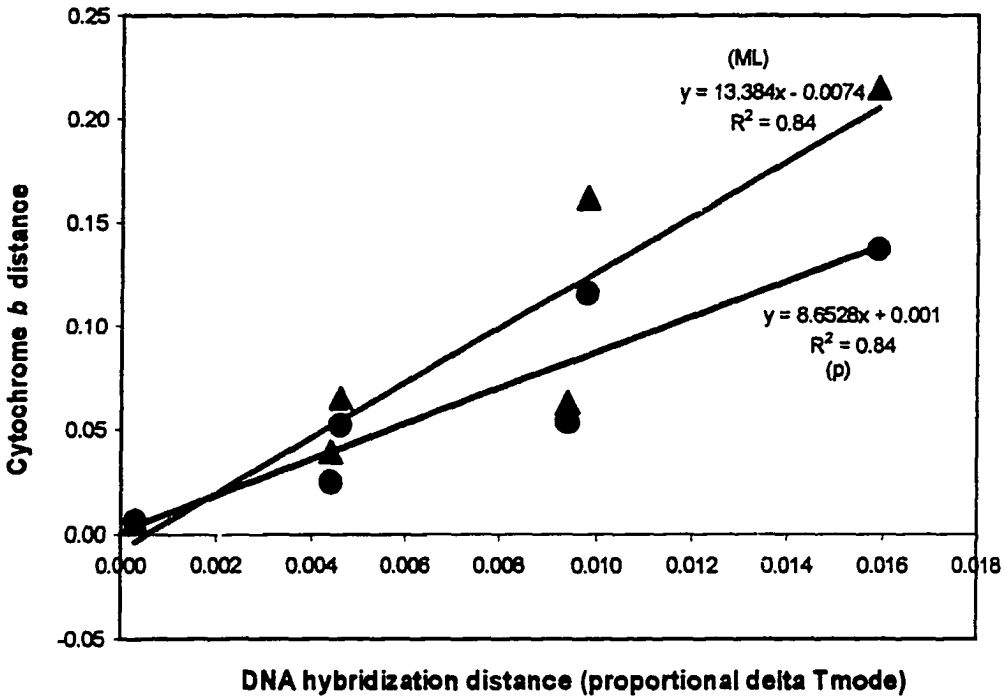

Figure 2.5---Partitioned plot of cytochrome *b* distances versus DNA hybridization distances; only unsaturated divergences are compared. Triangles represent maximum likelihood distances (ML); circles represent uncorrected proportional distances (p).

partitioned plot extrapolates the highest proportional delta Tmode value (0.0428, divergence between *T. minor* and *T. glis*) to a proportional cytochrome *b* distance of 0.3703. This value lies close to the highest maximum likelihood distances (0.3686, Table 2.2), suggesting that both maximum likelihood distances and DNA hybridization distances are not compressed. The slope of the regression between maximum likelihood and DNA hybridization distances in full scaling (7.91, Figure 2.4B) is very similar to that of the regression between the proportional cytochrome *b* and DNA hybridization distances in the partitioned plot (8.65, Figure 2.5). This similarity again suggests that the maximum likelihood correction is not compressed. This finding is unexpected given the slight flattening on the right tail of the maximum likelihood-DNA hybridization curve in the full scaling (Figure 2.4A).

Interestingly, the regression between maximum likelihood and DNA hybridization distances in the partitioned plot indicates that cytochrome *b* evolves 13.38 times faster than the scn-DNA among taxa that have diverged relatively recently (Figure 2.5). The maximum likelihood estimate differs substantially from the phenetic estimate of p-distances (see above, 13.38 versus 8.65). These two estimates are expected to be similar given that the partitioned plots only compared unsaturated divergence. Paradoxically, these two different estimates converged to the similar genetic divergences (see above, ML distance 0.3686 vs. extrapolated p-distance 0.3703).

One possible explanation for this discrepancy is that the maximum likelihood estimate exhibits a greater variance among taxa that have diverged relatively recently because increasing the parameters in the model (10 in GTR+I+Γ model) increases

the error in point estimation (Swofford et al. 1996). Or, alternatively, this discrepancy may be the result of stochastic fluctuation in estimation due to a small number of data points.

Estimates of phylogeny

The distribution of tree length produced by the exhaustive search was left-skewed with a g_1-value of -0.6850 ($p<0.01$), suggesting that the data set contains a substantial amount of phylogenetic signal. Maximum likelihood analysis of cytochrome *b* sequences using the GTR+I+Γ model produced one optimal tree (Figure 2.6). The log-likelihood (Ln) value was -5187.89; the tree length was 684. When different ratios of Ti:Tv (1:1 to 9:1) were used, the parsimony method yielded three tree topologies with *Urogale* shifting around different branches. In an equally weighted parsimony tree (Ti:Tv=1:1), *Urogale* was the sister group of *T. tana*: (((((*Urogale*, *T. tana*), *T. montana*), *T. minor*), *T. gracilis*), *T. glis*). When the ratio of Ti:Tv was set to 2:1 and 3:1, parsimony yielded a topology similar to that of the maximum likelihood tree, with *Urogale* as a sister group to the clade of *T. montana* and *T. tana*. The Ti:Tv ratios of 4:1 through 9:1 yielded only one topology, in which *Urogale* was the sister group of the clade consisting of *T. montana*, *T. tana*, and *T. minor*: (((((*T. montana*, *T. tana*), *T. minor*), *Urogale*), *T. gracilis*), *T. glis*). This topology is similar to that of the DNA hybridization tree rooted with *T. glis* (Figure 2.7). Because the cytochrome *b* data are saturated, I excluded the equally weighted parsimony tree (Ti:Tv=1:1) from subsequent topology comparisons.

When examined using the test of Kishino-Hasegawa (1989, Table 2.3), the maximum likelihood tree based on cytochrome *b* data (Figure 2.6) and the DNA

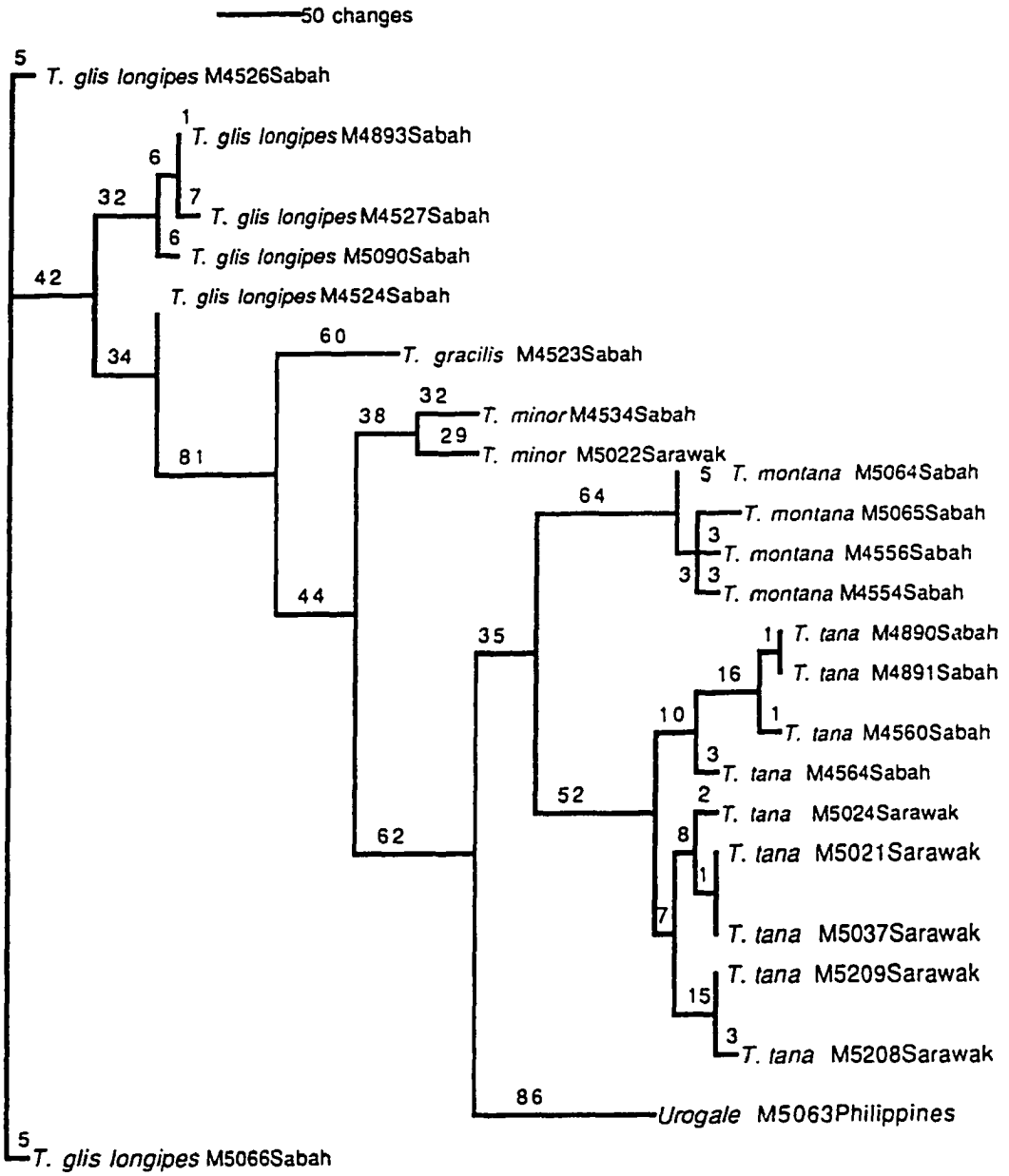

Figure 2.6—Phylogram of six tree shrew species derived from cytochrome *b* sequences based on the maximum likelihood model of GTR+I+Γ with the following rate-parameters: A-C 1.8301, A-G 5.2067, A-T 1.6081, C-G 0.1519, and C-T 9.3035; α=3.399, and I=0.5895. Ln=-5187.89. Numbers on branches indicate branch lengths in nucleotide changes.

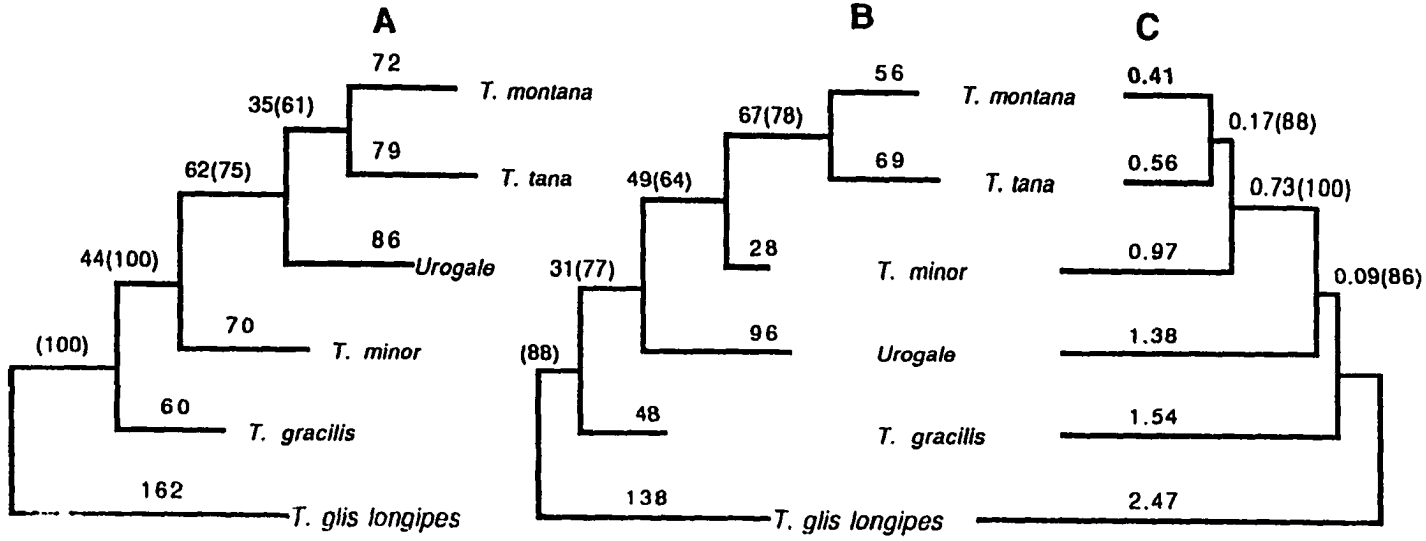

Figure 2.7---Phylogenetic hypotheses of six tree shrew species. (A) and (B) are cytochrome *b* trees derived from all codon positions and third-position transversions, respectively, using a maximum likelihood model of GTR+I+Γ. (A) is a simplified phylogram of Figure 2.6, showing only intraspecific individuals with the longest branch length. Rate-parameters for (B) were A-C 0.1955, A-G 39.0412, A-T 0.0031, C-G 0.1059, C-T 31.7093; α=0.2354, and I=0.0084. (C) DNA hybridization tree, built using the least-squares option of the program "Fitch" in PHYLIP version 3.4 (Felsenstein 1989) from delta Tmode values (Han et al. 2000). Numbers indicate branch lengths in nucleotide changes for cytochrome *b* trees and in Delta Tmode values for DNA hybridization tree. Numbers in parentheses indicate the proportion of 100 bootstrap trees supporting each branch.

Table 2.3---Topology tests of various phylogenetic hypotheses using the method of Kishino-Hasegawa (1989) under the framework of maximum likelihood.

	Kishino-Hasegawa's test	
Tree/comparisons*	-ln L	P value
ML tree	5187.89	
(vs.) DNA-*Urogale*	5229.46	0.0053**
(vs.) DNA-*T. glis*	5196.11	0.3199 (ns)

*DNA-*Urogale* and DNA-*T. glis* refer to the DNA hybridization trees rooted with *Urogale* and *T. glis* respectively

**difference is significant at $p<0.05$

ns = non-significant

hybridization tree rooted with *Urogale* (not shown) are significantly different ($p<0.05$) from each other in topology. However, the maximum likelihood tree is not different from the DNA hybridization tree rooted using *T. glis* (Figure 2.7C). This test clearly rejects *Urogale* as the outgroup of *Tupaia*. In fact, in all parsimony trees and the maximum likelihood tree, *Urogale* consistently lies within the *Tupaia* clade.

Although the maximum likelihood tree and the DNA hybridization tree rooted with *T. glis* are not significantly different, these two trees are not entirely congruent (Figure 2.7A vs. 2.7C). However, the incongruence seems to be superficial, reflecting the difficulty of deciphering closely timed branching points around the divergence of *Urogale*. Or, alternatively, the incongruence may reflect the weaknesses of each tree-building technique. Whereas the maximum likelihood correction seems to perform well at high interspecific levels (ML distances >0.25), it performs less well at lower level of divergence. This is evident from the bootstrap value (Figure 2.7A), which decrease markedly from 100% at the root of the tree to 75% on the branch leading to *Urogale*, and finally to 61% on the branch leading to the sister group of *T. montana*, and *T. tana*. Correction with the GTR+I+Γ ML model appears to resolve the hierarchical structure of deep branches at the expense of shallow branches. On the other hand, the DNA hybridization tree efficiently resolves the distal clade consisting of *T. minor*, *T. montana*, and *T. tana* with a bootstrap value of 100%. This clade is congruent with the immunodiffusion tree of tree shrews (Dene et al. 1978). However, the base of DNA hybridization tree was unclear because there was no outgroup available for rooting. Basal positioning of taxa had to be clarified with maximum likelihood examination of cytochrome *b* data.

Because the third-position transversional distances of the cytochrome *b* data yield unsaturated maximum likelihood distances (Figure 2.3), I constructed another tree using only third-position transversions to investigate the taxonomic congruence between cytochrome *b* and DNA hybridization data. The third-position transversions yielded a tree-length distribution g_1 value of -0.6702, which is very similar to that using all-positions with transitions and transversions combined (g_1=-0.6850), suggesting that much of the phylogenetic signal came from the third-position transversions.

The phylogeny based on third-position transversions assumed the same maximum likelihood model, GTR+I+Γ, as the tree based on complete data (Figure 2.7B). Rate-parameters were re-estimated using PAUP*. The tree, bootstrapped using 100 pseudo-replicates, yielded the same topology as the DNA hybridization tree rooted with *T. glis* (Figure 2.7 B vs. C). The log-likelihood value was -5196.11; the tree length was 711. Thus, complete taxonomic congruence between cytochrome *b* and DNA hybridization trees was attained only after the cytochrome *b* data were largely, if not completely, cleared of noise. The bootstrap value increased from 61 to 78 for the *T. montana-T. tana* clade in the cytochrome *b* tree based on third-position transversions, as opposed to the tree based on all-positions (Figure 2.7 A vs. B). However, the bootstrap value decreased from 100 to 88 at the basal branch joining *T. glis* to the rest of the species.

Comparison of the cytochrome *b* tree based on third-position transversions and the DNA hybridization tree (Figure 2.7 B vs. C) indicates that lineage-based rate variation is more pronounced in the cytochrome *b* data. The lineage-based rate is

slowest in *T. minor* and *T. gracilis* and moderately slow in *Urogale* cytochrome *b* data, as opposed to the DNA hybridization data. Both data sets indicate a rapid lineage-based rate of evolution in *T. glis longipes.*

In summary, the DNA hybridization data indicate that the maximum likelihood distills most of the phylogenetic signal from the cytochrome *b* data. However, for complete congruence between the two methods, only third-position transversions of cytochrome *b* can be used. Also, there is lineage-based rate variation among the tree shrews, especially in their cytochrome *b* gene. Both data sets support the position of *Urogale* as a member of *Tupaia* rather than as a separate genus.

CHAPTER 3

A NEW SPECIES OF BUSHY-TAILED TREE SHREW (SCANDENTIA: TUPAIIDAE: *TUPAIA*) FROM SARAWAK, MALAYSIA

INTRODUCTION

On 31 July 1997, while collecting for a small mammal inventory of Lanjak-Entimau Wildlife Sanctuary (LEWS) at an elevation of 260 m near the Engkari river, Ulu Engkari, Sarawak, under sanction of the Sarawak Forestry Department, I trapped two lactating adult female tree shrews on the ground. These two specimens, similar in body size to the montane tree shrew, *Tupaia montana*, resembled the common tree shrew *T. glis* in coat coloration, but were substantially smaller and less brownish. I also noted marked differences in fur texture and pelage color between these individuals and *T. montana* and *T. glis*. Given that *T. montana* is generally restricted to habitats above 900 m elevation, and that a typical female *T. glis* specimen was trapped 1.5 km upstream from the Engkari river locality on 26 July 1997, these two specimens seemed to represent a new species. A male specimen of this form was obtained on 2 November 1997 near Serembuang river, Ulu Skrang, LEWS, at the same elevation. In addition to elevation, these three specimens differed qualitatively from *T. montana*, which has longer, darker green, and fluffier fur.

There are five extant genera recognized within Tupaiidae (*Anathana*, *Dendrogale*, *Ptilocercus*, *Tupaia*, and *Urogale*), representing ca. 16 species. *Tupaia* is the largest and most widespread genus with ca. 11 species. Morphologically, most tree shrew species are similar, showing few differences in external characters. The genera are separated almost exclusively on the basis of soft cranioskeletal features. However, to a certain extent, the genera are recognizable externally by their tail

patterns and are divided into three groups: the pen-tailed tupaiid (*Ptilocercus*), the smooth-tailed tupaiids (*Dendrogale*), and the bushy-tailed tupaiids (*Anathana*, *Tupaia*, and *Urogale*). Species within *Tupaia* are distinguished by a combination of skull and body measurements, pelage color, the presence or absence of a longitudinal mid-dorsal stripe, habitat, elevation (lowland or montane), and geographic locality (Medway 1977, Payne et al. 1985, Han et al. 2000). In this paper, I contrast this apparent new species with other tupaiids by morphometric and genetic analyses. These analyses, combined with pelage features and elevations, indicate that the specimens from LEWS constitute a new species.

MATERIALS AND METHODS

Morphometric comparisons

Three voucher specimens are stored in Sarawak Museum, Kuching, and catalogued under the International Tropical Timber Organization (ITTO) small mammal series. Only two skulls were extracted. Body mass (Wt), in grams, and four other standard measurements, in mm, were taken: head and body length (HB), tail length (T), hind foot length (HF), and ear length (E). Using calipers, six cranial measurements as defined by Davis (1962) were recorded to the nearest mm: greatest length (GL), basal length (BL), intra-orbital length (IOB), palatal length (PL), zygomatic breadth (ZB), and orbital-snout length (OS). The new species was compared to four other medium-sized and/or slender-bodied tree shrews from Borneo: the striped *T. dorsalis*, *T. glis*, and *T. montana*, and the painted tupaiid, *T. picta*.

Molecular comparisons

Given the small sample size of the unknown taxon and its substantial overlap in skull and body measurements with *T. montana*, multivariate analysis of morphometric variation might not be able to distinguish it from *T. montana*. Moreover, prior allozyme analysis of 20 presumptive loci encoding for 15 enzymes did not yield fixed allelic differences or species-specific diagnostic loci among *T. montana*, *T. tana*, *T. glis longipes*, and *T. minor* (Han 1991). Thus, I used cytochrome *b* DNA sequence variation to help determine the validity of this taxon as a species.

Tissue handling, DNA extraction, PCR amplification, sequencing, and data analysis.---Tissues were available for two specimens only. Liver, heart, kidney, and thigh muscle tissues were collected from freshly killed specimens and stored in liquid nitrogen. Eight species were compared in this study. Except for *Urogale everetti*, specimens analyzed were generally collected over a broad geographic distribution in Borneo (Figure 3.1, Appendix). In addition to the six tree shrew species that were sequenced in Chapter 2, another species, *Tupaia dorsalis*, was also sequenced. Methods of PCR amplification, sequencing, and sequence alignments are essentially the same as those in Chapter 2, except that an annealing temperature of 51°C was used during PCR amplification for the new taxon.

All individuals were sequenced completely in both directions for the entire cytochrome *b* gene. Sequence alignments were unambiguous because insertions and deletions were absent. Uncorrected pairwise genetic distances were calculated from the aligned sequences of 1140 characters using PAUP* 4.0 (Swofford 1998).

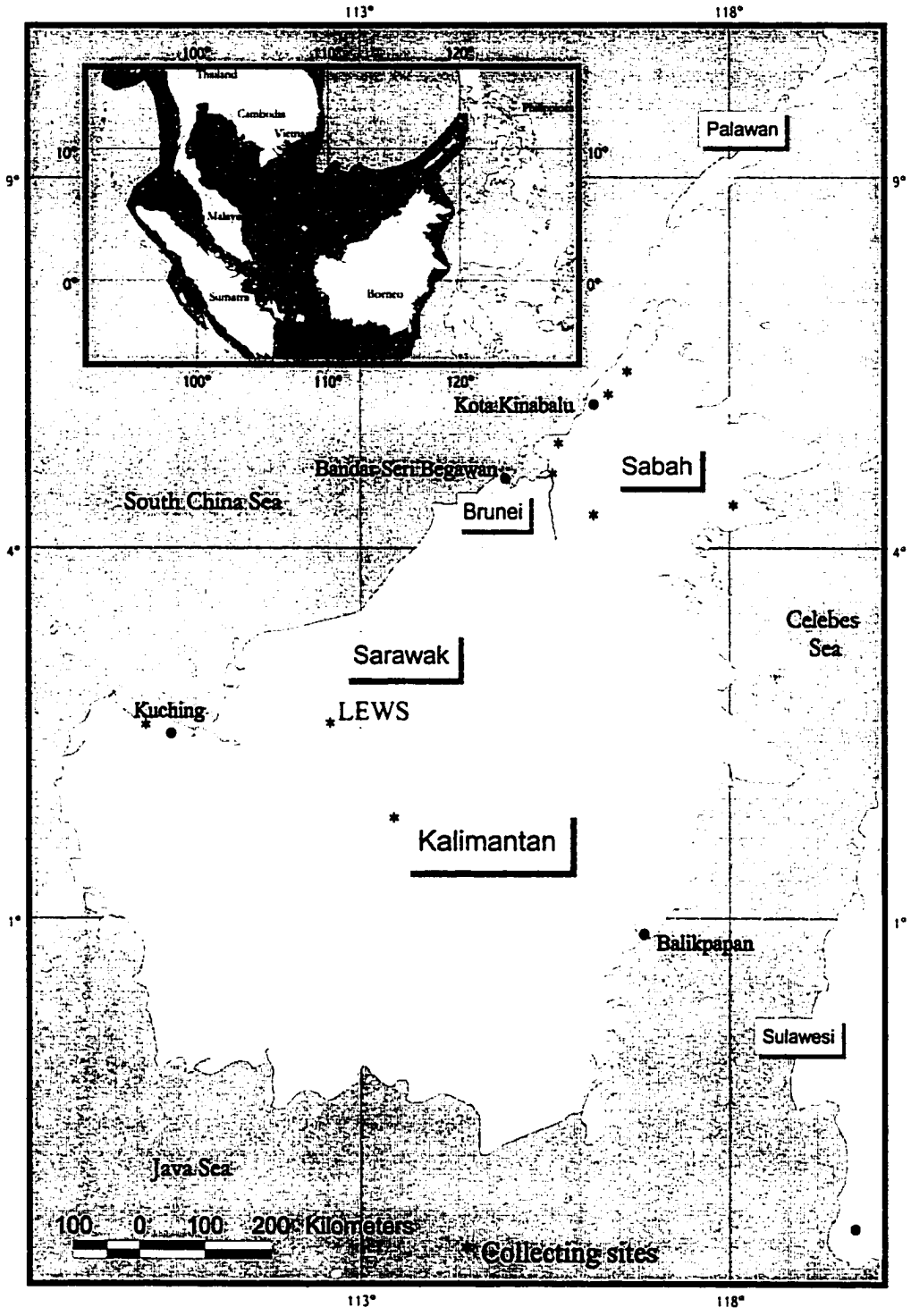

Figure 3.1---Collecting sites in Borneo.

RESULTS

Tupaia stuebingi, new species

Holotype.---ITTOM78; an adult female trapped on 31 July 1997 by the author in a relatively undisturbed primary forest at an elevation of 260 m on the ground at Ulu Engkari, 1 km downstream and opposite the ITTO field station, near coordinates 111°58'E and 1°25'N, Lanjak-Entimau Wildlife Sanctuary (LEWS), Sarawak, Malaysia. This specimen is a study skin, with a complete skull and mandibles in good condition. Soft tissues, including heart, liver, kidney, and muscles, were also sampled and frozen in liquid nitrogen.

Paratypes.---Adult female (ITTOM77) collected simultaneously at the type locality, preserved in fluid with soft tissues extracted; ITTOM123, adult male collected 2 November 1997, near coordinates 111°56'E and 1°32'N, Serembuang river, Ulu Skrang, LEWS, preserved in fluid, with skull extracted, but soft tissues were not preserved.

Habitat.---Located between 111°51'E and 112°30'E and 1°19'N and 1°51'N in the interior of Sarawak, LEWS covers ca. 168,758 ha (Figure 3.1). Bounded by the Bentuang-Karimun National Park (Kalimantan) on the southeast and the Batang Ai National Park (Sarawak) on the south, LEWS is drained by two of Sarawak's major river systems, the Batang Lupar and the Rejang. The Batang Lupar consists of a network of streams, including the Engkari, Lubang Baya, and Skrang rivers in the south. In the north, Katibas, Bloh, Mujok, Ensireng, Kanowit, and Ngemah rivers flow into the Rejang. This network of streams appears to have acted as a natural barrier to the spread of many small mammal populations. Ecologically, LEWS

sustains a wide diversity of climax vegetation, including 50 to 100 years old secondary forests, alluvial forest, gallery forest, mixed lowland and hilly dipterocarp primary forest, and submontane mossy forest. Its highest peak is Mount Lanjak (1,280 m). Interestingly, montane mammals are yet to be found in LEWS (Han 1998). Given that an elevation of 1,000 m is the lower limit for the distribution of the montane tree shrews in Borneo (Han et al. 2000), LEWS appears to be a refugium that may have been isolated from other montane zones of Sarawak in the past.

Distribution.---The new species is known only from the southern part of LEWS.

Etymology.---I have the pleasure of naming this new species for Robert B. Stuebing in recognition of his many contributions over the last 20 years as a teacher and researcher of vertebrate biology, natural history, and biodiversity in Borneo.

Diagnosis.---*Tupaia stuebingi*, a medium-sized, slender-bodied tree shrew, possesses a smooth, fine textured, olive-green coat on its upper parts when alive (i.e., when its fur is not combed). The olive-green fur is blended somewhat sparsely with light green and brownish hairs. However, when the skin ages, the fur on the upper parts changes into a dark black with gray roots. Its under parts, with four mammae, are yellowish white. A short, black, longitudinal, mid-dorsal stripe lies on its upper parts, stopping just behind the shoulder by blending uniformly into the background color. The shoulder streaks, yellowish white in color, are narrow and inconspicuous except on close examination. The tail is of intermediate length, with a T/HB ratio of approximately 1. The tail is thickly haired throughout its length, broad, bushy,

squirrel-like in character, and uniformly greenish in color. The snout is rather short compared to other species.

Description and comparisons.---Table 3.1 compares morphometric variation, coat coloration, and fur texture of *T. stuebingi* to four other *Tupaia* species found in Borneo. The smooth, olive-green coat of *T. stuebingi* appears to be closer to the brownish-green of *T. glis* in texture than to the light green coat of *T. gracilis* and *T. minor*. Like *T. glis*, *T. stuebingi* appears to be a lowland, semi-arboreal, forest-dwelling species, although further verification is needed. These characteristics are in contrast to the fluffy, terrestrial, short-tailed *T. montana* (T/HB<1), which is commonly found in disturbed montane forests above 900 m. *T. dorsalis* is light brownish in pelage color with a characteristic longitudinal mid-dorsal stripe, and *T. picta* has a characteristic rufous tail. *T. gracilis* and *T. minor* are lowland species, arboreal, long-tailed (T/HB>1), and substantially smaller in body size than *T. stuebingi*. The aged voucher skins of *T. stuebingi*, devoid of any greenish tinge, resemble the black upper parts of the *T. tana* skins, but without any reddish shades. When compared with the Javan tupaiid (*T. javanica*) and the Palawan tupaiid (*T. palawanensis*), *T. stuebingi* is substantially larger than the former but smaller than the latter, particularly in skull measurements (data in Han *et al.* 2000).

Genetic analysis.---The uncorrected pairwise cytochrome *b* distances among *Tupaia* and *Urogale* species ranged from 11.10% to 18.69% (Table 3.2). Within species, the cytochrome *b* distances for *T. stuebingi*, *T. montana*, *T. glis longipes*, *T. minor*, and *T. tana* were 0.18%, 0.62%, 5.20%, 5.35%, and 2.47%, respectively.

Table 3.1---Morphometric variation (in mm or grams), pelage and coat coloration, and upper part features of five tree shrew species from Borneo.

	T. stuebingi (n=3)*	*T. montana* (n=71)	*T. dorsalis* (n=3)	*T. picta* (n=2)	*T. glis longipes* (n=24)
HB	162.7±11.0	167.2±14.2	155.3±13.5	185	203.5±17.6
range	152–174	112–195	135–173	178–192	172–295
T	155.7±6.7	148.5±11.6	139.0±4.6	160.5	191.3±10.8
range	148–160	127–192	135–144	152–169	160–210
HF	41.3±2.1	38.6±1.9	42.7±2.3	41.5	49.7±1.8
range	39–43	34–42	40–44	41–42	45–54
E	12.3±0.6	15.5±1.7	13.7±0.6	15.5	14.9±1.8
range	12–13	12–20	13–14	15–16	11–18
T/HB	1.02±0.09	0.88±0.07	0.86±0.02	0.87	0.95±0.02
range	0.91–1.09	0.86–0.95	0.83–0.87	0.85–0.88	0.79–1.13
Wt	110.3±16.8	110.5±25.0	109.3±9.3	-----	162.2±26.3
range	92–114	72–145	103–120		95–200
GL	48.6	48.0±1.6	43.9±4.5	50.4	51.4±2.1
range	47.7–49.4	43.6–52.4	39.2–48.1	48.6–52.1	46.4–55.0
IOB	14.5	14.4±0.8	12.5±1.6	13.9	15.0±0.5
range	14.2–14.7	13.3–15.2	10.6–13.7	13.4–14.3	14.2–15.7
ZB	24.2	25.1±1.1	20.2±2.8	24.1	26.6±1.0
range	23.3–25.0	22.2–27.0	17.1–22.6	23.4–24.8	24.4–28.0
PL	23.7	25.8±1.1	24.2±3.6	28.0	28.3±1.8
range	23.2–24.1	23.6–28.5	27.3–20.2	26.5–29.4	24.0–30.7
BL	42.3	42.4±1.5	38.9±4.0	44.8	46.1±2.0
range	41.9–42.7	37.7–45.7	34.8–42.8	42.8–46.7	40.9–49.9
OS	21.8	21.9±1.5	22.4±4.1	24.1	22.0±1.9
range	20.9–22.6	17.7–28.4	18.2–26.3	22.5–25.6	17.4–24.7
coat coloration	olive-green	darker greenish	light brownish	reddish brown	green brownish
mid-dorsal stripe	short, stops after the shoulder	no or not distinct	conspicuous, continue through the rump	no or not distinct	no or not distinct
shoulder streaks	faint, yellowish white	faint	conspicuous, whitish	no or not distinct	conspicuous, whitish
fur texture	fine, smooth	longer, fluffier	fine, smooth	long, rough	freckled, fine, smooth

*n=2 for GL, IOB, ZB, PL, BL, and OS

DISCUSSION

As expected, the present study shows that *T. stuebingi* and *T. montana* have the shortest uncorrected pairwise cytochrome *b* distances (11.10%) among the tree shrews compared. A phylogenetic reconstruction using maximum likelihood indicates that *T. stuebingi* and *T. montana* are sister species (Chapter 4). Among the taxa compared, there is an obvious discontinuity in genetic distances among and between species. Intraspecific distances are ≤5.35% and interspecific distances are ≥11.10%. This discontinuity suggests that there has not been gene flow among species, and *T. stuebingi* seems to be well-differentiated.

A lack of gene flow between *T. stuebingi* and its sister taxon, *T. montana*, is manifested in their apparently different ecological requirements as well as genetic distances. These two species differ in at least three respects: *T. stuebingi* appears to be a lowland, semi-arboreal, and probably rare species occurring mainly in undisturbed primary forests, whereas *T. montana* is a terrestrial species, commonly found in disturbed montane forests. The genetic and ecological differences between *T. stuebingi* and *T. montana* suggest that they do not interbreed and hence are biological species (Mayr 1963).

In addition to ecology and genetics, *T. stuebingi* and *T. montana* are distinguishable morphologically based on fur texture, and more notably, in the ratio of tail length to head and body length (T/HB). *T. stuebingi* appears to have a T/HB ratio approximately 1 or slightly greater than 1, whereas T/HB of *T. montana* is substantially lower than 1. A tail of intermediate length (T/HB approximately 1) has often been considered to be the ancestral state for tree shrews, whereas both short-

Table 3.2---Cytochrome *b* sequence divergence for eight tree shrew species.

	1	2	3	4	5	6	7	8
1. *T. stuebingi* (n=2)	0.18							
2. *T. montana* (n=4)	11.10	0.62						
3. *T. dorsalis* (n=2)	18.64	17.19	1.23					
4. *T. glis longipes* (n=6)	16.18	18.69	18.38	5.20				
5. *T. gracilis* (n=1)	17.85	16.54	17.72	16.62	---			
6. *T. minor* (n=2)	14.28	13.76	17.76	17.25	13.33	5.35		
7. *T. tana* (n=9)	12.52	11.61	16.59	17.30	15.83	13.71	2.47	
8. *Urogale everetti* (n=1)	16.71	15.77	18.51	17.60	17.54	17.50	14.72	---

and long-tails are viewed as derived states (Martin 1968). If Martin's (1968) assumption is correct, then *T. stuebingi*, with an ancestral tail length, and *T. montana*, with a derived tail length, would each form a diagnosable evolutionary unit and, in view of the phylogenetic species concept (Cracraft 1983), would constitute different species.

The discovery of *T. stuebingi* underscores a peculiar relationship between body size and geographical distribution of *Tupaia* species. On a regional scale, three general patterns are evident. First, species that are unequivocally distinguishable based on body size often coexist on the same island or in the same geographic area, and often in sympatry. For example, *T. glis longipes*, *T. montana*, *T. minor*, and *T. gracilis* in Borneo; *T. glis glis* and *T. minor* in Malaya; *T. javanica*, *T. tana*, and *T. glis* in Sumatra; *T. javanica* and *T. glis* in Java. Second, species that cannot be distinguished based on body size measurements are generally allopatric. For example, *T. javanica* in Java and Sumatra, *T. montana* in Borneo, *T. palawanensis* in the Philippines, the species complex of *T. glis* (*belangeri* in Thailand, *chinensis* in Vietnam and Yunan, *glis* in Malaya, and *longipes* in Sabah), *T. picta* in north to central Borneo, and the ruddy tupaiid, *T. splendidula*, in southeast Kalimantan are all the same size. Third, if species overlap in body size and if they are found on the same island or in the same geographic area, then they are ecologically delineated, and hence not synoptic. This is the case of *T. stuebingi* and *T. montana*. This relationship between body size and geographic distribution, coupled with the fact that *Tupaia* species are generally similar in other morphological characters, suggests

that body size is the primary attribute involved in interspecific differentiation among species.

The discovery of a new species of tree shrew in Borneo highlights the fact that our knowledge of vertebrate biodiversity, particularly in central Borneo, is incomplete, despite more than 100 years of exploration. It is critical that we continue to document the biodiversity in Borneo as rapidly as possible considering that the pace of deforestation has quickened at an unprecedented rate, leaving ever-diminishing pristine habitats available to wildlife. In particular, LEWS, as a refugium sustaining no fewer than 58 species of scansorial mammals and 30% of the mammals endemic to Borneo (Han 1998), should be protected at all cost.

CHAPTER 4

MOLECULAR SYSTEMATICS AND HISTORICAL BIOGEOGRAPHY OF THE TREE SHREWS (TUPAIIDAE) BASED ON CYTOCHROME *B* SEQUENCE VARIATION

INTRODUCTION

Patterns of regional species diversification often reflect the relative importance of underlying biogeographic processes (Thorpe et al. 1994). For example, recently colonized regions may exhibit low levels of genetic variability, if the colonization event involved only a few founding individuals. Conversely, taxa from ancestral regions are likely to be paraphyletic with respect to more broadly distributed, derived taxa in recently colonized regions during a range expansion process (Templeton 1994). Therefore, examining the concordance between phylogeny and the fragmentation of a species' range may help identify the association of clades with geographic boundaries and the biogeographic processes that coincided with the formation of the boundary. When organismic molecular evolution is interpreted in light of the historical events that shaped the present-day geography of an area, it can be used to reconstruct the origin and the sequential divergences among closely related species. Thus, a phylogenetic framework becomes indispensable for understanding historical biogeography and speciation.

The tree shrews, or tupaiids (Tupaiidae), in the Malay Archipelago, or Sundaland, are an excellent mammal group with which to examine the geographical structuring of molecular differentiation as a basis for clarifying species limits. First, the tree shrews, by virtue of their phylogenetic history, are enigmatic and intriguing at various taxonomic levels: interordinal, intrafamilial, and subspecific. Their

relationship to other mammal orders is unclear; they show little morphological variation among species within the family; and many of their presumed intraspecific geographic populations have diverged to the extent of being different species.

Second, island populations of tree shrews within the Malay Archipelago are geographically isolated, and this isolation has played an important role in the evolution of new species. Tree shrews have a long history dating back to at least the mid-Oligocene (ca. 25-30 million years ago, Han et al. 2000), offering the opportunity to investigate lineage differentiation following both deep and more recent changes in biogeography. Because the tupaiids attain their highest diversity on the island of Borneo, my attempt to clarify their regional diversification and taxonomy in this chapter focuses on their colonization of that island.

Palaeogeological evidence (Metcalfe 1988 and references therein) indicates that the huge landmass of Borneo is a composite structure, consisting of at least two terranes formed by the accretion of continental plates during Palaeozoic and Mesozoic. As a result of this accretion, northern Borneo (Sabah) is geologically distinct from southwestern (Sarawak) and southern (Kalimantan) Borneo. In addition, Borneo has a complex history of land bridges repeatedly connecting and disconnecting it from its neighboring islands (Java, Palawan, and Sumatra) and mainland Asia (the Malay Peninsula). The connection and disconnection of these islands, coupled with the separation of Asian (Sunda) and Australian (Sahul) continental plates at the eastern edge of Borneo (Wallace's line), generated a land bridge speciation pump. The numerous highlands (up to 4,100 m at Mount Kinabalu) and several large river systems were natural barriers to the spread of many

small mammals, including tree shrews. By virtue of the speciation pump and its unique geography, Borneo developed an unusually rich diversity of vertebrates. This diversity exhibits a complex pattern of widespread and endemic taxa, a pattern that is particularly evident among the tree shrews.

One tree shrew species of special interest in historical biogeography is the common tree shrew, *Tupaia glis*. It is a polytypic species that traverses many geographical boundaries throughout Southeast Asia. At least five of its presumed subspecies have been recognized as putative species (Lyon 1913, Chasen 1933, Dene at al. 1978, Martin 1984): *chinensis* in Yunan and Hainan (China), *belangeri* in Indochina, *glis* in the Malay Peninsula, *longipes* in Sabah, and less frequently, *salatana* in Sarawak. These five taxa are generally differentiated by small differences in body size and pelage color. One exception is *belangeri*, in which the female has three pairs of teats (Martin 1984), as opposed to two. The species status of these taxa has been the source of long-standing debates (e.g., Napier and Napier 1967, Martin 1984, Nowak 1991). The only study that investigated the taxonomic structure of the *Tupaia glis* species complex was the immunodiffusion analysis of Dene et al. (1978), who studied four of the five taxa. Dene et al. (1978) recognized all four taxa, *belangeri*, *chinensis*, *glis*, and *longipes*, as full species, although they had some reservations as to whether *chinensis* has attained complete reproductive isolation from other taxa (Dene et al. 1980). However, Dene et al. (1978, 1980) did not examine the biogeographical implications of their immunodiffusion data.

Previous study has suggested that Tupaiidae in Borneo might have dispersed from the Asian mainland and undergone *in situ* speciation (Han et al. 2000). To

investigate this possibility, I compare cytochrome *b* sequences among several species of tree shrews in this study at various taxonomic levels: population, intrafamilial, and interordinal. I test whether the diversity of the tupaiids, and of *T. glis* in particular, within Borneo are due to divergence *in situ*, i.e., whether the tupaiids and *T. glis* invaded Borneo just once before it was disconnected from the Asian mainland, or if Sabah and Sarawak were invaded separately. If the colonization happened just once, molecular differentiation among tupaiid populations of Sabah and Sarawak would be weakly structured geographically because the invasion would be relatively recent, i.e., occurring after the terranes of Sabah and Sarawak were sutured. In contrast, if the tupaiids colonized Sabah and Sarawak separately, there would be a strong association between clades and geographic boundaries, with each clade forming a monophyletic group. If the second scenario is true, then we would expect a deep split within the tupaiids of Borneo because the invasion might have occurred before the composite structure of Borneo was established. I examine if the mainland populations of *T. glis* are ancestral to the isolated Bornean populations. Also, I assess whether the cytochrome *b* sequences generated in this study provide adequate phylogenetic signal to resolve the interordinal relationships of tree shrews relative to other eutherian mammals.

MATERIALS AND METHODS

Selection of taxa

Taxa analyzed in this study include all those used in Chapters 2 and 3 plus the following species and putative species: *Dendrogale melanura*, *Ptilocercus lowii*, *Tupai picta*, *T. belangeri*, *T. chinensis*, *T. glis*, and *T. salatana*. Species, sample

sizes and sample numbers are given in the Appendix. Cytochrome *b* sequences of *T. belangeri* are those of Schmitz et al. (2000, GenBank accession number AF217811).

DNA extraction

Soft tissues were used for DNA extraction for all taxa except *T. picta*, *Dendrogale melanura*, and *Ptilocercus lowii*, for which museum study skins were used. Laboratory protocol for extracting DNA from tissue was essentially the same as in Chapter 2. For museum study skins, samples were extracted in a separate building from the primary molecular laboratory. All equipment and laboratory surfaces were cleaned with a 10%-bleach solution prior to extractions. Only filtered pipette tips were used. All reagents were exposed to UV light (254 nm) for at least 20 minutes prior to use. Extractions were done in sets of six, including five samples and a blank control extraction. Each finely chopped sample of museum study skin was added to 750 µl digestion buffer consisting of 37.5 µl of 200 mg/ml Dithiothreitol (DDT), 37.5 µl of 20% SDS, 75 µl of 10 mg/ml Proteinase K, and 600 µl of STE (10 mM NaCl, 10 mM Tris, and 2 mM EDTA). Each sample was then incubated overnight at 55°C in a rotating stand. Following Proteinase K digestion, three standard phenol-chloroform extractions were performed. Each supernatant was then placed on a Centricon 30-column (Millipore), and 1-ml of sterile, UV-treated water was added to each column. The columns were spun at 5,000 rpm in a Sorvall centrifuge for 20 minutes. An additional 2-ml of sterile, UV-treated water was added to each column, and the columns were centrifuged again in the same manner as before. Finally, the columns were reversed and spun at 3,000 rpm for 10 minutes to collect the concentrated sample. Each sample was brought to a final volume of 160

µl using sterile, UV-treated water that had been preheated at 65°C for 10 minutes to deactivate any DNAase. Each sample was aliquotted into four tubes of equal volume and stored at –20°C.

PCR amplification and DNA sequencing

PCR reactions and primers used for amplifying DNA extracted from tissues were the same as those in Chapter 2. The annealing temperatures were 50°C to 51°C. Because of the characteristic state of degraded DNA in historic samples, routine amplification of fragments exceeding 200 base pairs was not possible for DNA extracted from museum study skins (F. Villablanca, personal communication). All primer pairs (Table 4.1) were therefore designed to generate amplicons shorter than 200 base pairs. Amplification conditions were essentially those of Chapter 2, except that 5 µl of 10% Bovine Serum Albumin (BSA) were added to each 50-µl reaction, and an annealing temperature of 55°C was used. In addition, the primers and dNTP's were added to the reaction when samples had reached 85°C to avoid formation of primer-dimers. Post amplification and DNA sequencing protocols for skin sample DNA were the same as in Chapter 2, except PCR products were electrophoresed in a 2% agarose gel for 1 hour prior to Qiagen cleaning.

Sequence alignments

All fragments were sequenced in both directions. Sequences were aligned using Sequencher 3.1 (Gene Codes Corporation) and MacClade 3.0 (Maddison and Maddison 1992) with sequences of human, lemur (*Lemur catta*), hedgehog, (*Erinaceus europaeus*), shrew (*Sorex araneus*), rabbits (*Lepus sinensis* and *Oryctolagus cuniculus*), and pig (*Sus scrofa*) downloaded from GenBank (accession

Table 4.1---Primer pairs for PCR amplification of DNA extracted from museum study skins.

Primer pairs*	Expected fragment size	Taxa amplified
L-14724 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' H-143 5'-GTATGGCTAGGAATAGGC-3'	143 base pairs	*P. lowii, D. melanura, T. picta*
L-14990 5'-CCATCCAACATCTCAGCATGATGA-3' H-259 5'-GGCATATGAAGAATATGGATGC-3'	154 base pairs	*P. lowii, D. melanura, T picta*
L-230 5'-GAGTCATCCGCTACCTTCACG-3' H-403 5'-CCTCAGAATGATATTTGTCCTCA-3'	177 base pairs	*P. lowii, D. melanura, T. picta*
L-379 5'-GCATTCATAGGATACGTACT H-567 5'-GAACGATTACTAATGCTGTA	188 base pairs	*P. lowii, D. melanura, T. picta*
L-640 5'-GACGCAGATAAAATCCCA H-811 5'-SGCAAATARGAARTATCATTC	171 base pairs	*P. lowii, D. melanura, T. picta*
L-798 5'-TCACATCAARCCAGAATG H-991 5'-CATGTTAGTGTAAGTAGGTC	183 base pairs	*T. picta*
L-976 5'TGAATTCTAGTAGCCGAC H-1052 5'AGGACTGAGGCTACTTGGC	76 base pairs	*D. melanura, T.picta*
L-1018 5'GGACAACCTGTAGAACAC H-16065 5'GGAGTCTTCAGTCTCTGGTTTACAAGAC	122 base pairs	*P. lowii, D. melanura, T. picta*

*With the exception of L14724 (Irwin et al 1989), L14990 and H16065 (Helm-Bychowski and Cracraft 1991), which were taken from the literature, other primers are named based on the position of their first tree shrew cytochrome *b* base pair. Functional primers are yet to be developed for the fragment between positions 567 and 640.

numbers given in the Appendix). Sequence alignments were unambiguous with no internal stop codons. However, there were several differences between my alignment of *T. belangeri* and those of Schmitz et al. (2000), even though we found the same number of sites (1140 base pairs) in the tupaiid cytochrome *b* gene. Schmitz et al's (2000) alignment had four gaps at positions 207, 461, 717, and 974, relative to the positions of my alignment. They also had an extra A at position 1137, which I deleted to fit my sequences because all my 41 sequences obtained from tissues were consistent in alignments in this position. I believe these differences in alignments are due to a difference in the choice of reference taxon, and that Schmitz et al. did not have a second tree shrew sequence for comparison. I consistently encountered three gaps whenever human and lemur sequences were included in the alignments, and I had to correct the gaps manually. However, no gaps were present if tree shrew alignments were first compared with pig, shrew, and rabbit sequences, particularly if the tree shrew sequences had been aligned among themselves before being compared to the reference taxa.

Data analysis

Computer packages MEGA version 1.02 (Kumar et al. 1993) and PAUP*4.0b1 (Swofford 1998) were used for data analysis. Base frequencies and uncorrected pairwise distances (p-distances) were calculated from the full data set (45 ingroup taxa) with MEGA using the option pairwise-deletion for missing data. Distances were corrected with PAUP* using the most appropriate maximum likelihood model gamma-GTR-invariable sites (GTR+I+Γ), which was determined using ModelTest (Posada and Crandall 1998) with the following parameters: A-C

0.9547, A-G 7.0036, A-T 1.4550, C-G 0.3411, and C-T 8.2468. The proportion of invariable sites (I) was 0.5308, and the shape parameter of the gamma distribution (α) was 1.3918.

Phylogeny reconstruction

For intrafamilial comparisons, the full data set of 45 ingroup taxa was pruned by removing redundant individuals within species to optimize computation time. In the end, 23 taxa were used to build a tree, none of them outgroups. The skewness of tree-length distribution was evaluated using G statistics (Hillis and Huelsenbeck 1992) implemented in PAUP* based on 10,000 random trees. The rate-parameters were re-estimated with ModelTest (Posada and Crandall 1998), which yielded the same model (GTR+I+Γ). The maximum likelihood tree was bootstrapped based on 100 pseudo-replicates.

For interordinal comparisons, 15 ingroup species and all eight outgroup species were included. Variability of cytochrome *b* by codon positions among the ingroup and the outgroups was examined using MEGA. For phylogenetic reconstruction, third-codon positions were excluded for two reasons. First, the combined first- and second-positions gave a higher absolute value of g_1 (-0.6594), suggesting more phylogenetic signal than when the third-positions were included (g_1 =-0.5685, $p<0.01$). Second, the third-position transversional distances yielded a saturated curve when plotted against the maximum likelihood distances (graph not shown), further indicating that the third-codon positions are noisy. Schmitz et al. (2000) also excluded the third-codon positions from their interordinal phylogeny reconstruction. My interordinal phylogeny was built assuming a maximum

likelihood model of GTR+I+Γ. To minimize the variance of estimation associated with each parameter, only four rate-parameters were used, which gave a better bootstrap resolution than when using six rate-parameters. Parameters were estimated with PAUP*, and the interordinal phylogeny was bootstrapped (100 replications).

RESULTS AND DISCUSSION

Characterization of cytochrome *b* data

For DNA extracted from fresh tissues, each aligned tree shrew cytochrome *b* sequence consisted of 1140 base pairs (bp). However, only partial sequences were obtained from museum study skins: 873 bp from *Dendrogale melamura*, 768 bp from *Ptilocercus lowii*, and 913 bp from *Tupaia picta*. Table 4.2a lists the percentage of variable and parsimony-informative sites and the average nucleotide composition of the cytochrome *b* sequences. The nucleotide composition was fairly evenly distributed at first-codon positions, but less so at second and third positions. The pattern conforms to the composition of other mammal cytochrome *b* sequences (Irwin et al. 1991), suggesting that no pseudo- or non-homologous genes were amplified.

Comparison of a larger number of taxa with greater pairwise sequence divergence caused the variable and parsimony-informative sites to increase substantially over the variability seen for six species in Chapter 2. Number of variable and parsimony-informative sites increased from 383 to 477 and from 322 to 421, respectively. The proportion of parsimony-informative sites increased from 13.04% (42) to 16.63% (70) in the first-position, and from 4.35% (14) to 5.22% (22) in the second-position. In the third-position, the number of parsimony-informative

Table 4.2---Variability of cytochrome *b* sequences partitioned by sites and codon positions. (A) Intrafamilial comparisons among 15 tree shrew species (45 taxa). (B) Interordinal comparisons among 15 tree shrew species and eight presumptive outgroup species.

(A) Intrafamilial comparisons

						Variable sites	Parsimony-informative sites
	A	T	C	G	Total sites	% (#)	% (#)
1st position	27.5%	22.9%	25.3%	24.3%	380	20.33 (97)	16.63 (70)
2nd position	20.8%	40.6%	24.3%	14.3%	380	7.76 (37)	5.22 (22)
3rd position	39.3%	19.0%	37.7%	4.0%	380	71.91 (343)	78.15 (329)
All positions	29.2%	27.5%	29.1%	14.2%	1140	Total (477)	Total (421)

(B) Interordinal comparisons

						Variable sites	Parsimony-informative sites
	A	T	C	G	Total sites	% (#)	% (#)
1st position	28.0%	23.7%	25.1%	23.3%	380	25.77 (158)	22 (110)
2nd position	20.5%	40.8%	24.5%	14.2%	380	14.03 (86)	9 (45)
3rd position	39.1%	19.9%	37.2%	3.9%	380	60.20 (369)	69 (345)
All positions	29.2%	28.1%	28.9%	13.8%	1140	Total (613)	Total (500)

sites decreased from 82.61% to 78.15% (Table 4.2a). Nucleotide substitutions in the third-position were saturated, unlike those in first- and second-positions. The increase in saturation with increased genetic divergences is even more dramatic when the outgroups are included (Table 4.2b). The proportion of parsimony-informative sites then increased to 25% for first-, 12% for second-, and 62% for the third-positions. The increase in variability among the codon positions that accompanies increases in genetic divergence has a profound effect on the estimation of phylogeny, particularly on the resolution of shallow branches (see below).

Estimates of phylogeny

Intrafamilial comparisons.---The interspecific proportional distances (p-distances) of the tree shrews ranged from 0.0800 to 0.1928; maximum likelihood (ML) distances, corrected using a GTR+I+Γ model, ranged from 0.1089 to 0.4656 (Table 4.3). Maximum likelihood comparison yielded one optimal tree, which is presented with *Dendrogale* as the root (Figure 4.1) and *Ptilocercus* as the root (Figure 4.2). The log-likelihood value was -7758.15; the tree length was 1476. *Urogale* was also used to root the tree, but PAUP* automatically assigned *Urogale* to an ingroup. When p-distances are considered in light of the tree rooted with *Dendrogale* (Figure 4.1), the shallow branching taxa roughly correspond to the distal taxa: *T. montana, T. stuebingi, T. tana, T. gracilis, T. minor, T. dorsalis*, and *T. picta*. The deep branching taxa refer to the basal taxa such as *T. belangeri, T. chinensis, T. glis, T. longipes*, and *T. salatana*. *Urogale* appears to be intermediate between the shallow and deep branching taxa.

Table 4.3---Cytochrome *b* sequence divergence of 15 tree shrew species (45 taxa). Above diagonal are maximum likelihood (ML) distances (GTR+I+Γ model); below diagonal are uncorrected p-distances. The upper row in the diagonal are intraspecific ML distances, and the lower row are intraspecific p-distances.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Ptilcoercus*	---	0.2149	0.2257	0.2422	0.1085	0.3456	0.3726	0.2254	0.3140	0.3549	0.3162	0.2740	0.1750	0.2418	0.1516
2 *Dendrogale*	0.1147	---	0.3168	0.4326	0.2316	0.3451	0.3504	0.1999	0.3559	0.4482	0.3867	0.4065	0.2621	0.3803	0.2990
3 *Urogale*	0.1250	0.1466	---	0.4042	0.2817	0.4007	0.3984	0.3430	0.3871	0.4144	0.3958	0.3825	0.3301	0.3646	0.2920
4 *T. gracilis*	0.1354	0.1764	0.1754	---	0.3615	0.3440	0.3885	0.3703	0.3782	0.4504	0.3879	0.2430	0.3447	0.3899	0.3347
5 *T. picta*	0.0800	0.1263	0.1446	0.1676	---	0.3004	0.3100	0.2562	0.3304	0.4029	0.3518	0.2464	0.1838	0.1880	0.1289
6 *T. belangeri*	0.1645	0.1598	0.1778	0.1629	0.1516	---	0.1089	0.2953	0.3631	0.3864	0.4363	0.3702	0.3858	0.3784	0.3629
7 *T. chinensis*	0.1767	0.1657	0.1784	0.1769	0.1603	0.0848	0.0065 0.0064	0.3026	0.3294	0.3752	0.4397	0.3895	0.4004	0.3836	0.3748
8 *T. glis*	0.1263	0.1158	0.1640	0.1732	0.1394	0.1574	0.1605	0.0182 0.0175	0.2740	0.3274	0.3657	0.3852	0.3561	0.3305	0.3450
9 *T. longipes*	0.1521	0.1654	0.1760	0.1662	0.1603	0.1749	0.1638	0.1480	0.0661 0.0520	0.1470	0.4128	0.3834	0.4337	0.4213	0.3665
10 *T. salatana*	0.1654	0.1822	0.1763	0.1836	0.1822	0.1772	0.1749	0.1658	0.1021	0.0371 0.0333	0.4656	0.4539	0.4496	0.4578	0.3810
11 *T. dorsalis*	0.1615	0.1845	0.1851	0.1772	0.1720	0.1913	0.1901	0.1724	0.1838	0.1895	0.0097 0.0123	0.3676	0.3818	0.3839	0.3321
12 *T. minor*	0.1491	0.1695	0.1750	0.1333	0.1397	0.1736	0.1716	0.1696	0.1725	0.1893	0.1776	0.0624 0.0535	0.2334	0.2454	0.2305
13 *T. montana*	0.1088	0.1329	0.1577	0.1654	0.1161	0.1723	0.1785	0.1677	0.1869	0.1928	0.1719	0.1376	0.0092 0.0062	0.1605	0.1731
14 *T. stuebingi*	0.1393	0.1701	0.1671	0.1785	0.1194	0.1681	0.1700	0.1615	0.1618	0.1914	0.1864	0.1428	0.1110	0.0009 0.0180	0.1879
15 *T. tana*	0.1036	0.1437	0.1472	0.1583	0.0936	0.1656	0.1706	0.1627	0.1730	0.1771	0.1659	0.1371	0.1161	0.1252	0.0362 0.0247

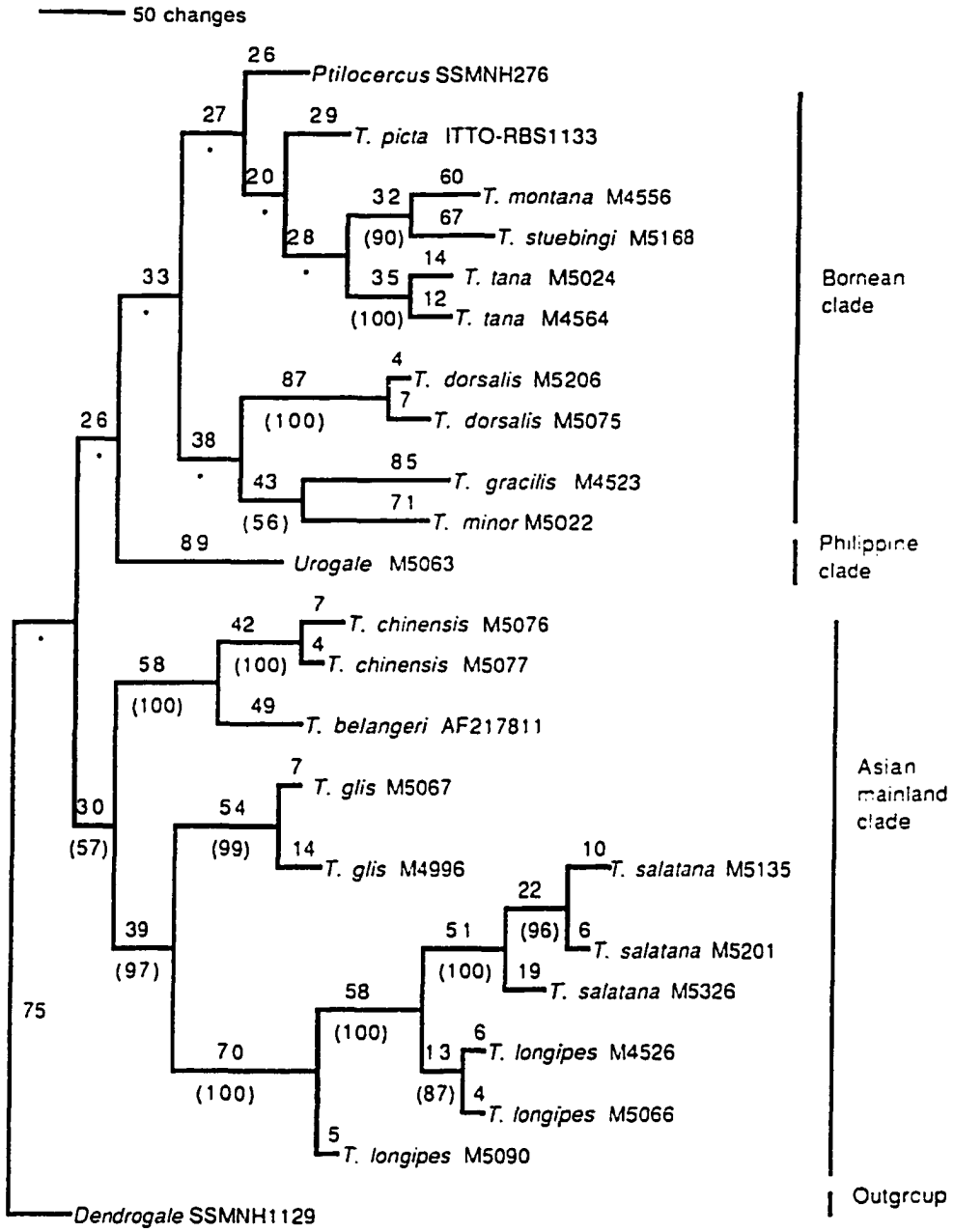

Figure 4.1---Maximum likelihood tree of cytochrome *b* for 15 tree shrew species based on the GTR+I+Γ model and rooted with *Dendrogale*. Rate-parameters were A-C 1.4738, A-G 9.5720, A-T 2.8925, C-G 0.4017, and C-T 13.6931; α=1.4292, and I=0.5315. Ln=-7758.15. Numbers on branches indicate branch lengths in nucleotide changes. Numbers in parentheses indicate the proportion of 100 bootstrap trees supporting each branch; * corresponds to <50% bootstrap values.

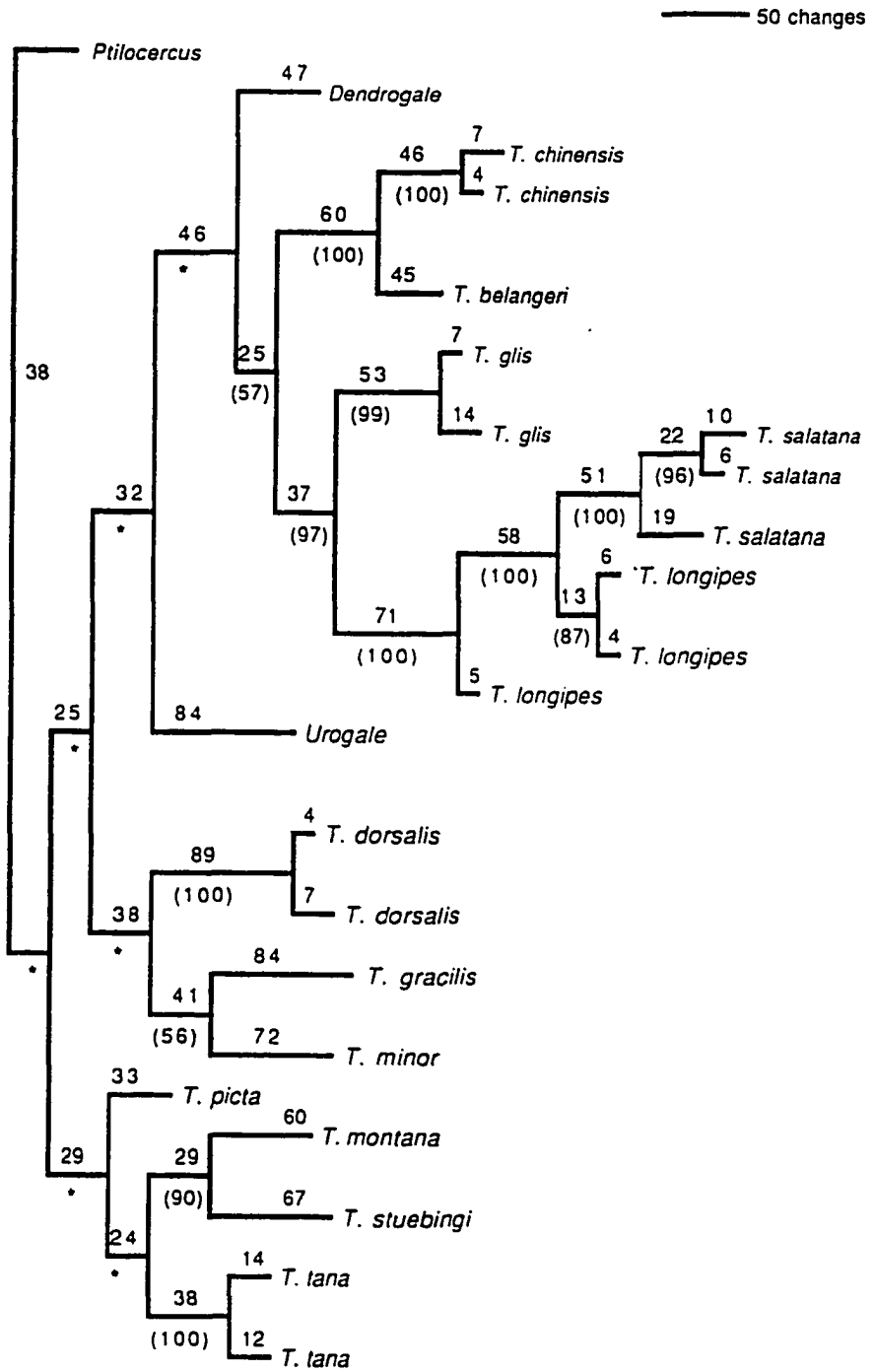

Figure 4.2---Maximum likelihood tree of cytochrome *b* for 15 tree shrew species based on the GTR+I+Γ model and rooted with *Ptilocercus*. Rate-parameters used, Ln value, and specimen numbers are the same as in Figure 4.1. Numbers on branches indicate branch lengths in nucleotide changes. Numbers in parentheses indicate the proportion of 100 bootstrap trees supporting each branch; * corresponds to <50% bootstrap values.

When bootstrapped, all branches of the maximum likelihood tree collapsed, except for the clade consisting of the *T. glis* species complex (Figures 4.1 and 4.2). Even within the *T. glis* clade, bootstrap values ranged from 100 at the terminal branch of *T. salatana-T. longipes* to 57 at the internal branch uniting *T. belangeri-T. chinensis*, and *T. glis-T. longipes-T. salatana*. The maximum likelihood GTR+I+Γ model appears to have supported basal branches at the expense of distal taxa.

Poor bootstrap resolution is unexpected, given that this data set contains a significantly high amount of phylogenetic signal (g_1=-0.7466), and that the six-species maximum likelihood tree (Figure 2.7A) had a logical structure taxonomically and biogeographically. The poor bootstrap resolution among distal branches appears to stem from the fact the tree shrews consist of a mixed array of taxa with drastically different histories, namely deep versus shallow time of divergence. The third-position changes are heavily saturated in both basal and distal taxa. However, given their larger divergences, first- and second-positions of the basal taxa have accumulated sufficient signal to overcome the noise caused by the saturation at third-codon positions. This is suggested by the slight decrease in the absolute values of g_1 from –0.7466 to -0.6596 when the third-positions were excluded from comparisons. For the distal taxa, their third-codon positions are as saturated as the basal taxa, but their first- and second-positions are not yet variable enough to provide adequate phylogenetic signal.

In an effort to improve the reliability of the maximum likelihood tree, third-codon positions were excluded during tree building. The attempt did not improve the resolution; the phylogenetic signal deteriorated with the absolute values of g_1

decreasing from -0.7466 (all positions) to –0.6596 (first- and second-codon positions). Rate-parameters of the GTR+I+Γ model were also reduced from six to four (two classes of transversions and two classes of transitions) to minimize the variance of the maximum likelihood estimate associated with each parameter. Again, the resulting tree remained poorly resolved when bootstrapped. This fruitless attempt suggests that a much longer sequence, such as the complete mitochondrial genome, would be required to derive adequate signal from the first- and second-codon positions to resolve the tupaiid phylogeny. Or, alternatively, one or more nuclear genes, with slower and more even rates of evolution, may be required to resolve the divergences among the deep- and shallow-branching taxa (DeFilippis and Moore 2000). Also, bootstrapping may be overly stringent (Hillis and Bull 1993) and incapable of providing realistic probability of branch support for the tree shrews.

Because there are no other tupaiid sequence data sets available for intrafamilial comparisons, I followed the approach of Sheldon et al. (1999) and inferred tupaiid phylogeny using a phylogenetic framework approach (Hillis 1987, Lanyon 1993), which in turn is based on combinable component consensus (Bremer 1990). According to this rationale, different components of trees from independent data sets are compared, and those parts of the trees that agree in branching order are accepted as phylogenetic hypotheses, whereas those that are inconsistent, based on bootstrapping, remain unresolved. Three tree components appear consistent based on this method. First, *Urogale* is a member of *Tupaia* and distal to *T. longipes*. This component is consistent between the DNA hybridization tree and the six-species cytochrome *b* tree (Chapter 2). Second, *T. stuebingi* is a sister taxon of *T. montana*

(bootstrap value 90, Figures 4.1 and 4.2), and these two species form the sister of *T. tana*. The *T. stuebingi-T. montana-T. tana* clade is distal as evidenced by immunodiffusion (Dene et al. 1978), DNA hybridization (Han et al. 2000), and the six-species cytochrome *b* trees (Figure 2.7A and C). Third, members of the *T. glis* complex form a monophyletic group, based on the immunodiffusion tree and the bootstrapped 15-species cytochrome *b* tree in the present analysis. Given these three consistent components, it appears that the tree rooted with *Dendrogale melanura* (Figure 4.1) is the most likely phylogenetic hypothesis for the tree shrews.

The tree rooted with *Dendrogale* shows a strong association between geography and phylogeny (Figure 4.1), suggesting that the tree shrews might have originated on the Asian mainland, probably with a center of radiation in Indochina. This dispersal pattern from north (Indochina) to south (the Malay Archipelago) is consistent throughout the tree in three respects. First, *Dendrogale* occurs on Indochina (eastern Thailand, Cambodia, southern Vietnam) and Borneo (Nowak 1991), whereas *Ptilocercus* is confined to the Malay Archipelago, i.e., Sumatra, the Malay Peninsula, and Borneo. The absence of *Dendrogale* on the Malay Peninsula is probably due to local extinction. The fact that *Dendrogale* is more widespread than *Ptilocercus* latitudinally suggests than *Dendrogale* is more ancestral. Second, the *T. glis* species complex showed a similar pattern, with Indochinese taxa (*T. chinensis* and *T. belangeri*) at the base of the clade. Third, the Bornean taxa are distal, suggesting that they are derived from the mainland taxa. In contrast, the tree rooted with *Ptilocercus* (Figure 4.2) is inconsistent in dispersal pattern. The basal clade suggests that the Asian mainland taxa are derived from Borneo, yet within the

Tupaia glis species complex the reverse is implicated (the Indochinese taxa, *T. belangeri* and *T. chinensis*, are basal to the Sundaland taxa, *T. glis*, *T. longipes*, and *T. salatana*).

Another argument for *Dendrogale* as the more likely basal taxon is based on the commonality principle that among alternative character states the state that is more common in distribution is plesiomorphic with respect to the more restricted (Frohlich 1987). *Ptilocercus* has a pen-tail or feather-tail, which is naked and dark except for the terminal part with whitish hairs on opposite sides, producing a feather-like form. This tail shape is rare among mammals, let alone among the tree shrews, and no tree shrews other than *Ptilocercus* have such an unusual tail. The grayish pelage of *Ptilocercus* is also unique among tree shrews, which normally have either greenish or reddish brown fur. Its huge ear with bare flaps, also stands out as unique among the tree shrews, as does its nocturnal habit. On the other hand, *Dendrogale* (*D. melanura* and *D. murina*) are smooth-tailed tree shrews, and are in many ways intermediate between *Ptilocercus* and other tree shrews both in the sparseness of the hairs on their tails and in their crepuscular habits (Martin 1984).

Interordinal comparisons.---Maximum likelihood comparisons among tree shrews and eight non-tree shrew outgroups yielded one optimal tree (Figure 4.3). The log-likelihood value was –11978.53; the tree length was 657. When bootstrapped, the interordinal tree provided better resolution among the tree shrew taxa than the intrafamilial tree (Figure 4.1). The improved robustness is not unexpected, since the data set now consists of more basal (eight presumptive

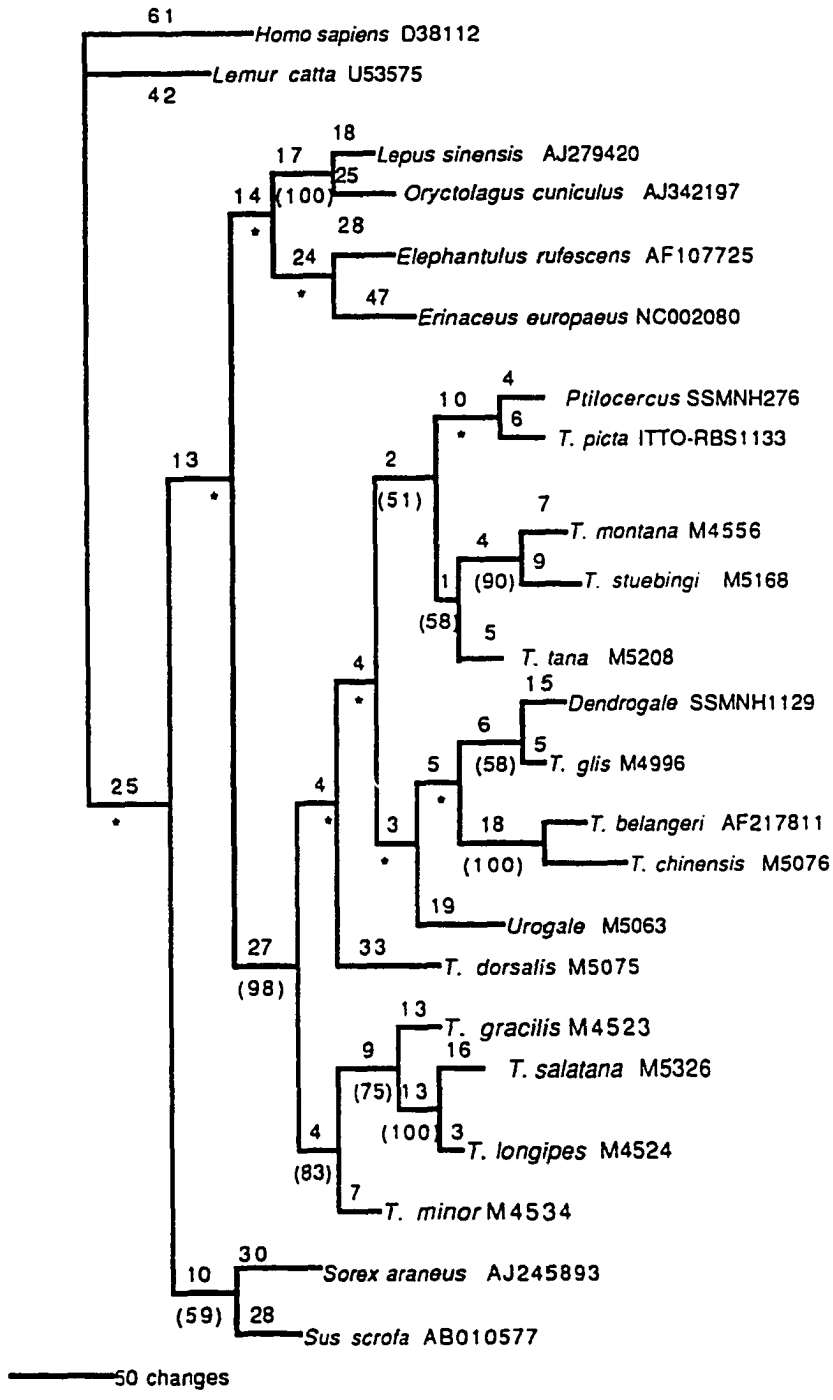

Figure 4.3---Interordinal maximum likelihood tree of cytochrome *b* for 23 mammal species based on the GTR+I+Γ model. Third-codon positions were excluded. Rate-parameters were A-C 1.1760, A-G 4.7885, A-T 1.0000, C-G 1.1760, and C-T 5.1248; α=0.6151, and I=0.5182. Ln=-11978.53. Numbers on branches indicate branch lengths in nucleotide changes. Numbers in parentheses indicate the proportion of 100 bootstrap trees supporting each branch; * corresponds to <50% bootstrap values.

outgroups and five taxa of the *T. glis* clade) than distal taxa and the model (GTR+I+Γ) estimates basal better than distal taxa.

The ingroup topology of tupaiids was contorted if one assumes that *Dendrogale* is the most likely basal tree shrew taxon), and that the *T. glis* species complex is monophyletic. The closest relative of tupaiids is a clade consisting of rabbits (*Lepus sinensis* and *Oryctolagus cuniculus*), hedgehog (*Erinaceus europaeus*), and elephant shrew (*Elephantus rufescens*). However, this sister grouping has a bootstrap value <50%. In terms of relative branch length (Figure 4.3), the number of nucleotide changes from the nearest common internal node for *T. belangeri* (68 changes) and *T. chinensis* (77 changes) is fewer than that of *Erinaceus europaeus* (98 changes), but more than those of *Elephantulus rufescens* (66 changes) and *Oryctolagus cuniculus* (56 changes). It should be noted that the tupaiid branch lengths have been cut substantially by the inclusion of another 13 ingroup species. Therefore, the affinity of tupaiids to the rabbit-hedgehog-elephant shrew clade may not reflect true phylogenetic relationships but is likely to be the result of long branch attraction between ingroup and distant outgroups (Felsenstein 1978, Hendy and Penny 1989, Wheeler 1990, Smith et al. 1992). Such ingroup-outgroup long branch attraction will be exacerbated if the longest ingroup branch is not basal (Smith et al. 1992), as in the case with tupaiids, where the distal taxa, *T. belangeri* and *T. chinensis*, have longer branches than the basal *Dendrogale* (64 changes). Thus, cytochrome *b* data are not adequate to resolve the interordinal relationships of tupaiids, and the sister group of tupaiids remains unclear.

The taxonomy of *Tupaia glis* species complex

Among the 45 taxa compared in this study, an obvious discontinuity in genetic distances exists between intra- and interspecific taxa. Uncorrected intraspecific p-distances are ≤5.35% and interspecific p-distances are ≥8.00% (Table 4.3). The ranges of intra- and interspecific differentiation among tree shrews conform to those found in other mammal groups, although the genetic distances of the tree shrews appear to be somewhat greater (Table 4.4). The pairwise p-distances among the taxa in the *T. glis* species group---*belangeri*, *chinensis*, *glis*, *longipes*, and *salatana*---range from 8.48% to 17.72%; these distances lie within the interspecific range (Table 4.4). The discontinuity from intra- to interspecific differentiation clearly indicates that there has not been gene flow among these five taxa, and they seem to be well-differentiated species. This finding is consistent with the immunodiffusion results of Dene et al. (1978), who recognized *belangeri*, *chinensis*, *glis*, and *longipes* as distinct species. *Tupaia salatana* was not examined by Dene et al. (1978), but based on differences in size and pelage color, Lyon (1913) and Chasen (1933) recognized it as a full species.

The genetic structure of the *T. glis* group is concordant with geographic boundaries. Also, differences in mating behavior have been observed during experiments in captivity. In captivity, no mating occurred among *T. chinensis* and *T. longipes* after 900 hours of observation (Conaway and Sorenson 1966). Reproductive isolation between the two taxa was attributed to differences in mounting postures, body size, periods of sexual receptivity, and agnostic behaviors of the females (Conaway and Sorenson 1966). Unfortunately, there are no other data available on

Table 4.4---Comparisons of cytochrome *b* sequence divergence among tree shrews and other eutherian mammals.

Intraspecific comparison			Interspecific comparison		
Taxon	% divergence*	Study	Taxon	% divergence**	Study
Tupaia spp. (tree shrews)	≤5.35	present study	Tupaiidae (tree shrews)	8.00 - 19.28 (p)	present study
Marmota spp. (marmots)	≤3.2	Steppan et al. (1999)	*Marmota* (marmots)	1.1 - 11.9 (p)	Steppan et al. (1999)
Phyllotis spp. (rodents)	7.6	Steppan (1998)	*Microtus* spp. (rodents)	1.5 - 18.0 (K2P)	Conroy and Cook (2000)
Pecari tajacu (peccary)	1.8	Theimer and Keim (1998)	*Akodontine* spp. (rodents)	5 – 20 (p)	Smith and Patton (1993)
Vulpes vellox (swift fox)	0.8	Mercure et al. (1993)	*Thomomys* spp. (rodents)	1 – 17 (p)	Patton and Smith (1994)
			Lagomorpha (rabbits)	2.39 – 25.26 (K2P)	Halanych and Robinson (1999)

* uncorrected p-distance.
** p = uncorrected p-distance.
K2P = distance corrected with Kimura's two-parameter model (Kimura 1980).

sexual behavior among *T. belangeri*, *T. glis*, and *T. salatana* with respect to *T. chinensis* and *T. longipes*. Even so, we can gain insight on the reproductive compatibility of the *T. glis* species group from their genetic divergences. The pairwise p-distance between *T. chinensis* and *T. longipes* is 16.38% (Table 4.3). Other pairwise comparisons within the *T. glis* clade lie close to this value, ranging from 14.80% to 17.72%, except for *T. belangeri-T. chinensis* and *T. longipes-T. salatana*, which are 8.48% and 10.21%, respectively. Therefore, the Bornean taxa are apparently well isolated genetically from the mainland taxa.

One interesting feature of genetic differentiation among the five taxa is that *T. belangeri* and *T. glis* have a relatively high pairwise p-distance of 15.74%, but are geographically connected by a narrow isthmus on the Kra Peninsula, with. *T. belangeri* occurring to the north (Indochina) and *T. glis* to the south (Malaya). Apparently, these species lie on two terranes with different geological origin (Metcalfe 1988). Their genetic divergence might also have been influenced by differences in climate associated with latitudes, since *T. belangeri* is confined to ca. 10°N to 17°N, whereas *T. glis* occurs between ca. 10°N and the equator. A change from a tropical (Malayan) climate in the south to a monsoon tropical climate in the north may have altered the selection pressure affecting each species.

As with *T. belangeri* and *T. glis*, *T. chinensis* and *T. belangeri* are connected geographically but have different geological origins and latitudinal ranges. *Tupaia chinensis* lies on the South China terrane, whereas *T. belangeri* lies on the Indochinese terrane. However, the latitude effect is probably less pronounced in *T. chinensis* and *T. belangeri* than in *T. glis* and *T. belangeri*. This is because *T.*

chinensis is generally confined to ca. 17°N and 22°N, with much the same monsoon climate as *T. belangeri*. This similarity in habitats and consequent selective pressures helps explain their low level of divergence (pairwise p-distance 8.48%).

The same can be said of the divergence between *T. longipes* and *T. salatana* of Borneo (pairwise p-distance 10.21%). Northern Borneo (Sabah) is geologically distinct from the southwestern Borneo (Sarawak and Kalimantan), the two areas being separated by a suture (Metcalfe 1988). To the north (above 4°N) is *T. longipes*, whereas *T. salatana* is generally confined to the south (below 4°N). These two species are exposed to the same tropical climate, but apparently prevented from interbreeding by the large river systems on Borneo. Alternatively, the two species may not have interbred yet because the terranes of Sabah and Sarawak might have been connected only recently.

Historical biogeography

Of the tree shrew taxa compared, the *Tupaia glis* species group provides the most information on historical biogeography, because its species are derived from both Borneo and the Asian mainland, and also because comparisons can be made between my data and the immunodiffusion data of Dene et al. (1978). The branching order of the *T. glis* group in the maximum likelihood tree of cytochrome *b* is quite different from that in the immunodiffusion tree (Figure 4.4). Because mainland taxa are distal in the immunodiffusion tree, they appear to have derived from Bornean taxa. Such a scenario is counter-intuitive, because Borneo has much higher tree shrew endemism. The immunodiffusion tree also implies that *Tupaia* might have originated in the Philippines, since the *T. glis* species group is distal to *Urogale*.

This geographic pattern is hard to reconcile with the fact that the basal taxon (*Dendrogale*) also occurs on the Asian mainland (Thailand, Cambodia, and southern Vietnam) and Borneo, but not in the Philippines. Moreover, many other studies suggest that the biota of Borneo is derived from the Asian mainland (e.g., Holloway 1997, Brandon-Jones 1998). It may be that the apparent backward topology of *T. glis* in the immunodiffusion tree results from a rooting problem (*Urogale* and *Ptilocercus*).

In contrast to the immunodiffusion tree, the maximum likelihood tree of cytochrome *b* depicts the Bornean taxa (*T. longipes* and *T. salatana*) are distal to the mainland taxa, *T. glis*, *T. chinensis*, and *T. belangeri* (Figure 4.4). Most strikingly, the Philippine tree shrew, *Urogale*, lies by itself, forming the sister group of the entire clade of Bornean taxa. The geographic association among clades is thus unequivocal: the basal clade consists of mainland taxa, the middle clade is the Philippine taxon, and the distal clade consists of Bornean taxa. In particular, the shape of the distal clade suggests a relatively recent adaptive radiation on Borneo and a rapid rate of molecular evolution among the tree shrews.

The phylogeny of tree shrews has several taxonomic implications. In the spirit of phylogenetic classification, *Urogale everetti* should be restored to its original name, *Tupaia everetti* Thomas, 1892, (Wilson and Reeder 1993) to make the genus *Tupaia* monophyletic. *Ptilocercus lowii* should also be renamed *T. lowii*, because it is one of the terminal taxa on the distal clade of *Tupaia* (Figure 4.1). This classification would be largely consistent with the character state tree of tupaiid tail shape: the ancestral smooth-tailed *Dendrogale*, the derived pen-tailed *Ptilocercus*,

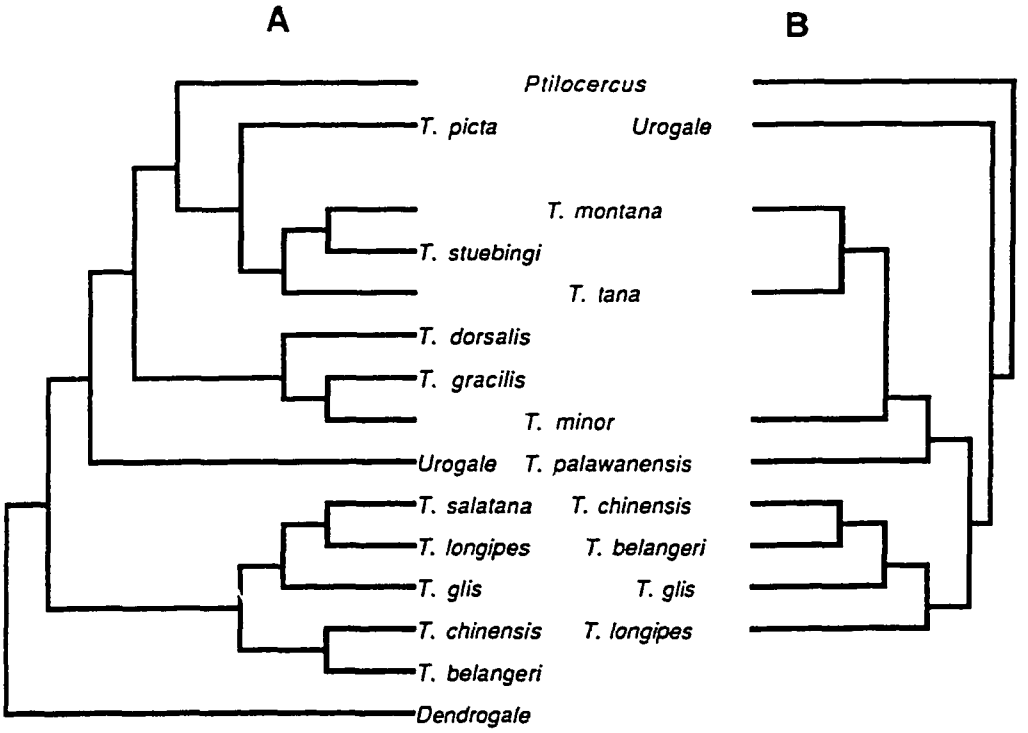

Figure 4.4---Comparison of taxonomic congruence. (A) Maximum likelihood tree of cytochrome *b* rooted with *Dendrogale*. (B) Immunodiffusion tree (Dene et al. 1978).

and the derived bushy-tailed *Tupaia*. If tail shape is a reliable indicator of ancestry, then the bushy-tailed Indian tree shrew, *Anathana ellioti*, should also be restored to its original name, *Tupaia ellioti* Waterhouse, 1850 (Wilson and Reeder 1993). In terms of biogeography, *A. ellioti* is similar to *T. chinensis* in that it occurs between ca. 17°N and 22°N. Of course, further data will be required to test this phylogenetic hypothesis of *A. ellioti*. It should be noted that *T. minor* and *T. tana* are each represented by a presumptive intraspecific population on the Asian mainland and Sumatra respectively. That these populations are conspecific seems unlikely, given the degree of differentiation in cytochrome *b* between all other island and mainland taxa, but the relationships still need to be tested genetically.

On a local scale in Borneo, the taxa of Sabah (*T. longipes*) and Sarawak (*T. salatana*) are structured in concordance with geography. Each forms a monophyletic group, thus supporting the hypothesis that the *T. glis* species group colonized Sabah and Sarawak on at least two separate occasions. Interestingly, the same geographic structure can also be seen at the intraspecific level: the *T. tana* population of Sabah (intraspecific p-distance 2.47%) is distinct from its Sarawak counterpart (Figure 2.6). The same is likely for *T. minor* (intraspecific p-distance 5.35%). Even though only one individual was sequenced for Sabah and Sarawak, these two individuals show a relatively long branch length from the internal node (Figure 2.6).

Two phylogeographic patterns are superimposed on each other in Borneo---one deep (between species) and one shallow (within species)---suggesting that the island was colonized not just separately but also repeatedly by species from the Asian mainland. The deep split between species, as exemplified by *T. longipes* and

T. salatana, seems to have occurred before the terranes of Sabah and Sarawak were sutured. These deep branching taxa would probably be the ancestors of *T. dorsalis*, *T. gracilis*, *T. montana*, *T. picta*, and *T. stuebingi*. On the other hand, the recent colonization, i.e., among the intraspecific populations of *T. minor* and *T. tana*, is probably a relatively recent event. The recent colonization was likely to have occurred after the present-day geologically composite structure of Borneo was established, thus allowing the intraspecific populations to interbreed. Unfortunately, we do not know the absolute date that the composite structure of Borneo was formed (Metcalfe 1988).

Although the southwestern terrane of Borneo (Sarawak and Kalimantan combined) is much larger in area than Sabah, its tree shrew populations do not seem to show strong intraspecific phylogeographic structure. Individuals of *T. dorsalis* from Sarawak (n=1) and Kalimantan (n=1) were weakly differentiated genetically (intraspecific p-distance 1.23%) with relatively short branch lengths from the internal node. However, it should be noted that these individuals were collected from two localities that were ca. 200 kilometers apart. Further data, including more extensive sampling from other species, particularly populations on Kalimantan, will certainly be required to resolve this issue.

CHAPTER 5

SUMMARY AND CONCLUSIONS

This dissertation examined the molecular systematics and historical biogeography of the tree shrews (Tupaiidae), encompassing intrafamilial and interordinal levels. The ingroup consisted of 15 species, including a new species (*Tupaia stuebingi*); eight other eutherian mammals were used as outgroups. The mitochondrial cytochrome *b* gene was the primary genetic marker. Comparisons were carried out between complete cytochrome *b* sequences (1140 base pairs) and single-copy nuclear DNA hybridization data for six tree shrew species to examine the relative rate of evolution between nuclear and cytochrome *b* DNA in tupaiids. The comparisons indicated that different rates have occurred among lineages, and that the Mindanao tree shrew, *Urogale everetti*, is a member of *Tupaia*. The cytochrome *b* and DNA hybridization data produced congruent phylogenies, suggesting that the two molecular methods are both phylogenetically informative.

Using ecological, morphological, and molecular data, I described a new species of *Tupaia* (*T. stuebingi*) based on three adult specimens from Lanjak-Entimau Wildlife Sanctuary, Sarawak. This lowland species is similar in size to the short-tailed montane species, *T. montana* (T/HB<1), except that its tail is of intermediate length (T/HB approximately 1). Its coat coloration and fur texture resembles the common species, *T. glis*. Cytochrome *b* sequence comparisons among eight taxa indicate that this species is most closely related to *T. montana* at a proportional distance of 11.1%.

Maximum likelihood comparisons among 15 tree shrew species yielded poor bootstrap resolution. Based on the logic of phylogenetic frameworks, the pen-tailed *Ptilocercus lowii* may in fact be a member of *Tupaia*, whereas *Dendrogale* is the more likely *Tupaia* outgroup. As such, *Ptilocercus* and *Urogale* should be included in the genus *Tupaia*. Such a classification scheme is consistent with the character state tree of the tupaiid tail shape: the ancestral smooth-tailed *Dendrogale*, the derived bushy-tailed (*Tupaia*), and the derived pen-tailed (*Ptilocercus*). If this hypothesis is correct, then the bushy-tailed *Anathana ellioti* should also be included in *Tupaia*. Also, all five taxa in the *T. glis* species group---*belangeri, chinensis, glis, longipes,* and *salatana*---should be recognized as full species in light of their large degree of genetic differentiation, morphological differences, and geographical separation.

There is a strong association between geography and the three major clades in the tupaiids: the basal clade consists of Asian mainland taxa, the middle clade of the Philippine taxa, and the distal clade of Bornean taxa. This pattern suggests that the Bornean tree shrews are derived from mainland ancestors. On a local scale in Borneo, two phylogeographic patterns are superimposed on each other---one deep (between species) and one shallow (within species)---suggesting that Borneo was colonized not just separately but also repeatedly by species from the mainland. The deep split seems to have occurred before the terranes of Sabah and Sarawak were sutured.

The sister group of tupaiids is a clade consisting of rabbits, hedgehog, and elephant shrew. However, this close affinity may not reflect their true phylogenetic

relationships but is likely to be the result of random long branch attraction between ingroup and distant outgroups. Thus, cytochrome *b* data are not adequate to resolve the interordinal relationships of tupaiids, and the sister group of tupaiids remains unclear.

Because of the poor bootstrap resolution of the maximum likelihood tree, future molecular studies of tupaiid phylogeny should use longer sequences, such as the complete mitochondrial genome, to derive sufficient phylogenetic signal from the first- and second-codon positions, or, alternatively, one or more nuclear genes, with slower and more constant rates of evolution.

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APPENDIX

Appendix---Species, sample sizes (n), DNA preparation numbers, and sampling localities.

Taxon*	Common name	n	Sampling locality	DNA preparation number**
1. *Tupaia longipes*	Common Sabahan tree shrew	6	Sabah: Lawa Mandau, Menumbok, Pensiangan, Tawau	M4893, M4524, M4526, M4527, M5066, M5090
2. *Tupaia gracilis*	Slender tree shrew	1	Sabah: Lawa Mandau	M4523
3. *Tupaia minor*	Lesser tree shrew	2	Sabah Lawa Mandau Sarawak: Kuching	M4534 M5022
4. *Tupaia montana*	Montane tree shrew	4	Sabah: Mendolong, Kiau, Kota Belud	M4554, M4556, M5064, M5065
5. *Tupaia tana*	Large tree shrew	9	Sabah: Lawa Mandau, Penampang, Sarawak: Kuching, Lanjak-Entimau	M4560, M4564, M4890, M4891, M5021, M5024, M5028, M5209 M5037
6. *Urogale everetti*	Mindanao tree shrew	1	The Philippines: Mindanao	M5063
7. *Tupaia dorsalis*	Striped tree shrew	2	Sarawak: Lanjak-Entimau Kalimantan:	M5206 M5075
8. *Tupaia stuebingi*	Stuebing's tree shrew	2	Sarawak: Lanjak-Entimau	M5167 (ITTOM077), M5168 (ITTOM078)
9. *Tupaia belangeri*	Belanger's tree shrew	1	downloaded from GenBank	AF217811

Appendix (Continued)

10. *Tupaia chinensis*	Common Chinese tree shrew	3	China: Guangxi	M5076, M5077, M5078
11. *Tupaia glis*	Common Malayan tree shrew	8	Malaya: Pahang (Temerloh)	M4994, M4995, M4996, M5067, M5069, M5070, M5071, M5072
12. *Tupaia salatana*	Common Sarawakian tree shrew	3	Sarawak: Kuching, Lanjak-Entimau	M5135, M5201, M5326
13. *Tupaia picta*	Painted tree shrew	1	Sarawak: Sibu area	ITTO-RBS1133
14. *Dendrogale melanura*	Smooth-tailed tree tree shrew	1	Sabah	SSMNH1129
15. *Ptilocercus lowii*	Pen-tailed tree shrew	1	Sabah	SSMNH276
16. *Homo sapiens*	Human	1	downloaded from GenBank	D38112
17. *Lemur catta*	Ring-tailed lemur	1	downloaded from GenBank	U53575
18. *Elephantulus rufescens*	Long-eared elephant shrew	1	downloaded from GenBank	AF107725
19. *Erinaceus europaeus*	Eurasian hedgehog	1	downloaded from GenBank	NC002080
20. *Sorex araneus*	European shrew	1	downloaded from GenBank	AJ245893

Appendix (Continued)

21. *Lepus sinensis*	Chinese hare	1	downloaded from GenBank	AJ279420
22. *Oryctolagus cuniculus*	Old world rabbit	1	downloaded from GenBank	AJ342197
23. *Sus scrofa*	Pig	1	downloaded from GenBank	AB010577

* Species 1-6 were used in comparison of cytochrome *b* and DNA hybridization (Chapter 2).
Species 1-8 were compared in describing the new species, *T. stuebingi* (Chapter 3).
Species 1-23 were compared in studying molecular systematics and historical biogeography of the tree shrews (Chapter 4).

** M = Lousiana State University Museum of Natural Science mammal tissue number.
SSMNH = Sabah State Museum Natural History number.
ITTO = International Tropical Timber Organization catalogue number; RBS = Rob B. Stuebing's catalogue number.

VITA

Kwai Hin Han was born on September 30, 1963 in Ipoh, Malaysia. He attended Universiti Kebangsaan Malaysia, Sabah Campus, in Borneo where he received his bachelor of science degree in biological sciences in 1988. He received his master of science degree from the same university in 1992. He is currently finishing his doctoral research in zoology at Louisiana State University and anticipates graduation in December 2000. He is married to Teck E Lim and has four children. Their youngest son was born on October 12, 2000 in Malaysia while Han was burning the midnight oil to finish his dissertation in Baton Rouge.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Kwai Hin Han

Major Field: Zoology

Title of Dissertation: Molecular Systematics and Historical Biogeography of the Tree Shrews (Tupaiidae)

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 26, 2000